Efficacy of essential oils against bacterial isolates from fresh water snails

BY
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CERTIFICATE - I

This is to certify that this thesis entitled, **"Efficacy of essential oils against bacterial isolates from fresh water snails"** submitted for the degree of **Master of Science** in the subject of **Zoology** to the Chaudhary Charan Singh Haryana Agricultural University, Hisar, is a bonafide research work carried out by **Ms. Jyoti Soni** under my supervision and that no part of this thesis has been submitted for any other degree.

The assistance and help received during the course of investigation have been fully acknowledged.

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CHAPTER-I

INTRODUCTION

Snails allied to molluscan Class Gastropoda and approximate 38 independent ancestry of gastropods have successfully pioneered the freshwater environments (Strong *et al.* 2011). Freshwater molluscs are the fundamental part of every aquatic ecosystem. They help in the ecosystem's functioning and also enable the ecologists to judge the health of their residence (Oloyede *et al.* 2017). Worldwide these shelled animals are used as the best biomonitoring tools. Snails are main invertebrates of the world as they are the second huge group of the animal kingdom after insects. These single-valved molluscs have branched out into every imaginable natural aquatic terrain including groundwaters, wetlands, streams, rivers, lakes and springs (Soldanova *et al.* 2013). They are generally found in man-made and ephemeral water bodies. Many species of snails spend their entire lives in a few square meters of habitat, making them exceedingly at risk to restrain environmental habitat decline. Freshwater snail species abundance is greatest in medium to large-size rivers with high dissolved oxygen concentrations, stable and clean substrates (Rao *et al*. 2002).

Evolutionary history of aquatic snails reveals that gastropods, cephalopods and bivalves developed in the Cambrian period about 541-585.4 million years ago. Gastropods naturally known as slugs and snails belong to freshwater, marine or terrestrial habitats (Lindberg, 2008; Loker, 2010; Khade and Mane, 2012). With more than 62,000 reported living species, they encompass about 80% of living molluscs and about 13,000 named genera (Okafor, 2009).

SNAILS stand for Self-Navigated Interleaved Spiral (Liu *et al*. 2004). Molluscs because of their wide-range, minute size and insufficient exploration have remained undiscovered or under described (Madhyastha *et al*. 2004). While globally, there has been a renewed interest in land snail research, in India the research has truly been at snail's pace (Aravind *et al.* 2005; 2010).

'Indian Malacology' was introduced by William Henry Benson and contributed remarkably to our awareness on Indian snails in the mid-19thcentury (Naggs, 1997). Near about 1488 species of snails belonging to 140 genera and 26 families have been recorded in India (Ramakrishna and Mitra, 2002; Madhyastha *et al.* 2004). From the phylum Mollusca many species are subjected to gene analysis efforts; many molluscs are chief food sources (snails, octopus and squids), also they can pass on parasitic diseases (Dayrat *et al*. 2011; Kocot *et al.* 2011).

Freshwater snails found in a variety of shapes, colour and size. Limpets are flat and simulate a capital building's dome, whereas Planorbids often have the shape of a ram's horn. The freshwater snails are very important from the medical and veterinary point of view as they are highly involved in the life cycle of parasites causing diseases to livestock and humans. Various chemical, physical and biological

factors affect the presence and the distribution of snails and thus, their combined effect influences a particular species or population (Ahmed, 2017; Kloos *et al*. 2001).

Molluscs are beneficial as they are highly visible, ecologically and commercially foremost source of food planetary and as non-food resources (Rittschof and McClellan-Green, 2005). Freshwater gastropods usually dominate stream subtidal in both mass and numbers (Cowie *et al*. 2009). Gastropods can serve as dietary element of several predatory animals, including turtles, fishes, crayfishes and ducks. They play a major role in nutrient cycling as they have an intense impact on algal primary productivity (Johnson, 2005). The snail species are important part of natural ecosystem as they play an important role as members of food chain (Ahmad *et al.* 2018). Nutritionists consider them as important source of high-quality protein, minerals, vitamin D and essential fatty acids including omega-3 fatty acids. Omega-3 fatty acids are involved in the prevention of cardiovascular diseases (Bodner-Montville *et al.* 2006; Sirot *et al*. 2008). Therefore, the national nutrition and health programme (PNNS) in France endorse consumption of these seafood twice a week especially for people who have heart attacks (Wardlaw and Kessel, 2002).

Many species of snails have medicinal importance. The factors utilized in this activity probably are the oleic acid and cyclopropane fatty acid rich lipid, isolated from the foot of this species (Bhattacharya *et al.* 2014). It can be used to treat illness including asthma, whooping cough, anaemia and high blood pressure due to their relatively low cholesterol level but high mineral content (Akinnusi, 2002). The consumption of snail meat can help stave off prenatal haemorrhage, osteoporosis, night blindness and hypophosphatemia; increase high density lipoprotein (Engmann *et al.* 2013). Borkakati *et al*. (2009) reported nine edible fresh water snail species in North East India. Freshwater molluscs play an important role in the economy and tradition of West Bengal (India) serving as a food in 81 percent families belonging to general scheduled, tribal people and person suffering from protein energy malnutrition. *Pila globosa, Melania tuberculate, Bellamya bengalensis* and *Lamellidens marginalis* are most edible gastropods of the region (Baby *et al*. 2010).

Soup prepared from the foot of fresh water edible snail (*B. bengalensis*) is traditionally consumed by the tribes of Jharkhand, India against rheumatism like bone and joint inflammation (Prabhakar and Roy, 2009).

With the decrease in per capital income associated with critical population pressure preferably in developing countries, increasing attention is now shifting to the consumption of new protein sources like edible mollusc flesh. Snail farming is an activity that involves management, production, harvest and sales of snails as well as a means of amplifying household income and protein supply (Okafor, 2009). It is a potential tool for food security as snails have the excellent nutritional potential and considering their environmental benefits the snails can be used as human food (Ghosh *et al*. 2016).

Snails collected from the uncultivated area can easily be kept in cages to grow and gain weight. In rural sections, snails are sold to supplement family income (Ebenso and Okafor, 2002; Ebenso, 2004).

The bacteria found in the gut of the gastro-intestinal tract (GIT) of aquatic snails are cellulose-degrading bacteria, lactic acid bacteria, proteolytic bacteria, chitinolytic bacteria and sulfate-reducing bacteria (Dar *et al.* 2017). According to Serrano *et al*. (2004) mesophilic aerobic bacteria *Enterobacteriaceae, Staphylococcus aureus* and coliforms are present among ready to eat snails. The coagulation of plasma of *S. aureus* along with their ß-haemolysis activity confirms their pathogenicity. This organism (though easily killed by boiling heat) produces enterotoxin that can tolerate heat at 100°C for 30 min and this toxin is known to cause food poisoning (Brook *et al*. 2004).

Pseudomonads are known to preponderate in the microflora of bivalves and gastropods from freshwater ecosystems (Syvokiene and Mickeniene, 2002). Micro-organisms isolated from the freshwater snail has health implications on man. The presence of *Escherichia coli* in the freshwater snail was an indication of secondary contamination. This secondary contamination was a result of sewage contamination of freshwater areas where harvesting the snails. *E. coli* was the causative agent of diarrhoea, dysentery, haemolytic uremic syndrome, septicaemia, pneumonia and meningitis (Kumar *et al*. 2005).

Snails may serve as intermediate hosts for parasites of humans and animals or can directly transmit disease. They are responsible for human schistosomiasis by acting as intermediate hosts of infective fluke (trematode) larvae of the genus *Schistosoma*, which are common in America, Africa and Asia (WHO 1993). Schistosomiasis ranked second parasitic disease (after malaria) in terms of its prevalence, socioeconomic and public health importance in tropical and subtropical areas (Chitsulo *et al*. 2000 and affects over 200 million people worldwide (King *et al*. 2005).

There is a very close association between snails and microbes because of their habit filth, sewage and rotten materials (Fagburo *et al.* 2006). Different enteric-pathogenic bacterial species ranging from *E. coli, Enterobacter, Proteus, Shigella, Aeromonas, Salmonella, Pseudomonas, Klebsiella* and *Staphylococcus* were present in the freshwater snail (*Ampullaria* spp.) in Abia state, Nigeria (Nwiyi and Amaechi, 2013). These pathogenic bacteria cause various bacterial diseases in carp culture in India like motile Aeromonads septicaemia, Edwardsiellosis, Pseudomonas septicaemia, Vibriosis, Bacterial gill disease (BGD), mycobacteriosis disease and enteric septicaemia (Mukherjee, 2002; Mohanty and Sahoo, 2007). Snails are thought to be a reservoir of these pathogenic bacteria, which is especially of importance among aquarium fishes (Decostere *et al.* 2004).

Due to monotonous and non-selective use of antimicrobial drugs by insufficient disease treatment, multiple drug resistance in microbial pathogens becomes a serious health problem to human kind worldwide (Peng *et al*. 2006). To obtain drug resistance, microbes have developed new enzyme

system to splinter the drug and make it useless for control of infection. Hence, plant origin herbal medicines are contemplated as safe substitutes of synthetic drugs. Conventional medicinal plants of eastern Uttar Pradesh (India), *i.e. Euphorbia pulcherima* and *E. hirta* (Family; *Euphorbiaceae*), *Thevetia peruviana* and *Alstonia scholaris* (Family; *Apocynaceae*) have formidable molluscicidal activity against freshwater snails that act as intermediate hosts for the larvae of trematodes, *Fasciola hepatica* and *F. gigantica* causing the diseases fascioliasis and schistosomiasis. These plant origin pesticides help in the removal of harmful snails from the ecosystem as they control the vector snails (Singh *et al*. 2010).

Monoterpenes are the vital constitutes found in essential oils (EOs) and they show potent antibacterial activity against carrier-related microflora found in freshwater snails. *Cannabis sativa* (Marijuana) and *Humulus lupulus* (common hop) essential oils are novel control tools for mollusc species. Both the oils are able to exert a good toxic effect against the prominent disease vector freshwater bladder snail *Physella acuta* (Draparnaud) (Mollusca *Physidae*). Myrcene, terpinolene and caryophyllene were the most profuse chemical components of *C. sativa* EO while in *H. lupulus* EO the major ingredients were humulene, myrcene and caryophyllene which was productive in killing the invasive snail *P. acuta* (Bedini *et al.* 2016).

It is, therefore, very important to assess the antimicrobial activity of herbs against bacterial pathogens present in snails, so that an effective control strategy could be evolved accordingly. Thus, keeping the above facts in view, the present investigation was carried out to screen the antimicrobial activity of essential oils against microbial agents isolated from fresh water snails with the following objectives.

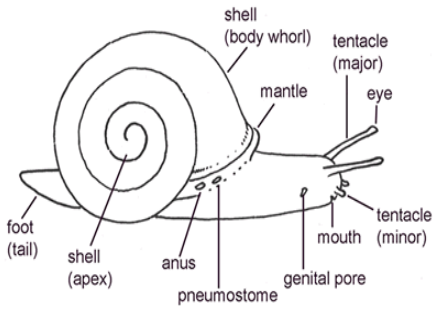
1) To isolate and characterize the bacterial isolates retrieved from fresh water snails
2) To evaluate the antibacterial activity of essential oils and antibiotics against bacterial isolates

CHAPTER- II

REVIEW OF LITERATURE

Freshwater snails are playing role in the field of scientific studies and research, since it has many ecological roles, having high nutritional values. Also, their ancient history, habits, territory, dissemination on local, regional and universal basin, their reproductive behaviour were studied. Hence, attempts have been made to present and review the available literature on these aspects. Snails are highly variegated aquatic molluscs and also play a vital role in agriculture. Hoverman *et al.* (2011) confirmed that discrepancy in territory conditions mediates species-classifying mechanisms and structures vernacular freshwater snail communities. Surveying of the species affluence, evenness and diversity showed that the fresh habitats had the highest species richness with the most manifold and even snail species conformation (Wang *et al*. 2015).

The snail's soft body is divided into three distinct sections. A well-defined head is connected to a large muscular foot. The foot is the most visible external feature of the snail's body and is the animal's mode of locomotion. A snail propels itself with this single foot, which is controlled by hydrostatic action inside the snail's body. Additionally, the foot surface is covered with tiny projections (cilia) that assist the gliding motion. Snails feed along the bottom as they move, partially clearing a path for the foot. As the foot passes over the substrate, it leaves a "trail" behind the snail. These trails are commonly visible in soft sediments or across hard surfaces such as rocks or submerged trees torsion (Crothers, 2012; Mackenstedt and Markel, 2001).



Source: http://www.thesnailwrangler.com/education/anatomy/

Snails play principal roles in the recycling of materials from the producers to the consumers' levels of the food chain in an ecosystem. Due to their litter feeding habits they recycle minerals from plant tissues to the soil thus improving soil fertility. Snails are equiped with a lot of enzymes including cellulases which allow them to play a major role in primary decomposition of plant materials. The mucoproteins in snail faeces and slime are useful in binding soil particles (Okafor, 2009). Vanni (2002) stated that animals can directly influence ecosystem-scale nutrient fluxes through excretion of biologically important compounds; foreign-born species may change nutrient fluxes by: (1) altering the biomass of consumers that recycle nutrients at given mass-specific rates (Hall *et al*. 2003), or (2) altering assemblages of organisms and their corresponding mass- and species- specific nutrient excretion ratios (McIntyre *et al*. 2008). Moslemi *et al*. (2012) concluded that invasive snail (*Tarebia granifera*) alter nutrient cycles mainly nitrogen cycle in aquatic systems in temperate and tropical zones.

In India, the taxonomic study on molluscs started more than two centuries ago (Benson, 1832). The Indian freshwater molluscs in the taxonomic literature had been found dissipate and sparse. Some of the significant ones are: Ray, 1951 (Coramendal coast of India); Ray, 1961 (South India); Roy and Gupta, 2010 (Barak River of Assam); Khade and Mane, 2012 (Ratnagiri, Maharashtra); Kumar and Vyas, 2012 (*Narmada* River); Biswas *et al.* 2015 (Kangra, Himachal Pradesh).

According to Rayate & Patil (2019), the fresh water gastropods existing in the three rivers viz. Godavari, Mula and Pravara of the Northern Region of Ahmednagar District, (M. S.) India, belong to a total six families from eight genera with eleven different gastropod species viz., *Bellamya dissimilis, M. tuberculata, B. bengalensis, Thiara scabr, Tarebia lineata, T. granifera, Indoplanorbis exustus, P. acuta, Lymnaea acuminate, L. luteola* and *Gyraulus convexiusculus* besides some species of bivalves. Freshwater gastropods had noteworthy ecological role in the marshy systems.

Marie *et al.* (2015) divulged that there is a direct association between water physico-chemical framework, trophic level of water and distribution of snails that have a medical consequence and maritime plants type. Over 650 species in North America are hinge from deep, interior Canada near the Arctic Circle to the subtropical regions of South Florida and Mexico. North America is the Earth's center of freshwater snail diversity (Johnson, 2005).

According to Wang *et al*. (2015) the freshwater natural surroundings of runnel, lagoon and rice paddies take over remarkably different abiotic water qualities, with temperature, water and pH showing clear-cut statistical differences ($P < 0.05$) and these different natural habitats had different snail multiplicity and species evenness, with high *Bithynia simensis* (goniomphalos snail) abundance at rice paddy habitats. The differences in snail wealth may be due to the well define sets of abiotic water qualities analogous with each habitat types. Kumar *et al.* (2019) revealed that *M. tuberculata* residing during pre-monsoon while *Filopaludina bengalensis* during post monsoon season. They collected total 359 gastropods both aquatic and land snails during their study from the *Morni* hills, Panchkula, Haryana and disclose that pre-monsoon season has greater Malacofaunal diversity (8.46 times) than post-monsoon. Seven genera, seven species and five families collected during the period.

Table 2.1: Classification of collected molluscan fauna from *Teekar Taal* during study period

Phylum	**Class**	**Habitat**	**Family**	**Genus**	**Species**
Mollusca	Gastropoda	Freshwater	*Viviparidae*	*Filopaludina*	*Bengalensis*
			Thiaridae	*Melanoides*	*Tuberculate*
			Planorbidae	*Gyraulus*	*Ladacensis*
				Indoplanorbis	*Exustus*
			Lymnaeidae	*Radix*	*Luteola*
		Terrestrial	*Ariophantidae*	*Ariophanta*	*Interrupta*
				Macrochlamys	*Indica*

Source: Kumar et al. (*2019)*

Neumann *et al*. (2002) reported that snails as nurture animal protein has elevated value of micronutrients like minerals, vitamins and essential amino acids. CICFRI (The Central Inland Capture Fisheries Research Institute) formulated a manual of *Achatina fulica* farming about three decennary ago. Heliciculture (snail farming) devote to the destitution reduction in Nigeria. Thus, it is a bonafide means of round off the carbohydrate meals of the inclusiveness of Nigeria as well as a resource of generating capital and to attain self-adequacy as it provides self-implementation to the people (Agbogidi and Okonta, 2011).

Four freshwater snails (*Brotia costula, B. bengalensis, B. dissimilis* and *P. globosa*) were assessed for proximate and mineral compositions as to conclude that freshwater snails of Tripura are rich in macro and micro nutrients needed for human body; they are safe from heavy metal contamination, hence recommended for regular consumption. Analysis of muscle tissues showed crude protein (11.18 to 15.59%), moisture (65.80 to 73.80%), lipid (0.82 to 1.15%) and carbohydrate (5.62 to 11.97%). Among the minerals, calcium (142 to 312.50 mg%), phosphorus (55.39 to 121.17 mg%), potassium (118.20 to 182.28 mg%), iron (4.03 to 68.64 mg%) and manganese (3.13 to 5.33 mg%) were reported (Debnath *et al*. 2016).

Table 2.2. Proximate composition of fresh snail meat

Nutrient	**Value**
Crude protein	18.20%
Carbohydrate	2.88%
Ether extract	1.36%
Fat	1.01%
Crude fibre	0.07%
Ash	1.37%
Nitrogen free extract	4.95%
Iron	12.2 mg/100g
Water	74.06%
Other minerals constituents	60.5m/100g

Source: (Agbogidi and Okonta, 2011).

The roll out of *Salvinia molesta*, a free-floating weed in tropical and sub-tropical regions of India is biologically administered by snails, especially *P. globosa* found in Kerala and other parts of India. It was dependent to feed voraciously on *Salvinia*, but not on paddy which forms a major crop in Kerala and seems not to be an intermediate host for liver fluke. The sum up of these advantageous characteristics, together with the fact that the snail has well organized adaptations for aquatic or terrestrial environments makes it suitable for *Salvinia* control. Snails can be considered beneficial bio-weeders (Joshi *et al*. 2005; Hidaka *et al*. 2007) in Agricultural wetlands. Snailery favoured to the poverty reduction in Nigeria as snail meat fulfilled with high nutritional values (Agbogidi and Okonta, 2011).

Bacterial diseases are the most devastating and widespread diseases of freshwater fishes and various antibiotics are used for the alleviation of these diseases e.g.; Penicillin, Tetracyclines,

Bacitracin, Streptomycin and Tylosin. Microflora have been conceded as assertive disease producing agent and includes *Pseudomonas* spp., *Aeromonas* spp., *Flavobacterium* spp., *Corynabacterium* spp., *Micrococcus* spp., *Staphylococcus* spp., *E. coli* and *Salmonella* spp. (Mohammed and Mailafa, 2010).

The freshwater aquarium snail (*Ampullaria* spp.) exhibits 108 feasible mesophilic bacteria per gram of meat and shell. Gram negatives preponderate in sixteen genera of bacteria. Enhancement culture techniques sanction the isolation of salmonellae from 24 of 42 lots of 200 g each. Including *Salmonella newport, S. saint-paul* and *S. infantis*, it embraces eight different serotypes. This association of salmonellae with snails contribute to cases in human salmonellosis. *Pseudomonas aeruginosa* and *Edwardsiella tarda* found occasionally. *Salmonella* infection is extensive in humans as organisms of the genus *Salmonella* which are comprehensively distributed pathogens in nature that causes salmonellosis in humans (Bartlett and Trust, 1976).

Temelli *et al.* (2006) resolute the major contagion sources during frozen snail meat refinement and inspect it for the recital of total aerobic mesophilic bacteria, *Listeria* spp., moulds and yeasts. The microbiological (epidemiological), imminent and mineral element composition was studied and found that the bacterial isolates were *S. aureus, Bacillus subtilis, E. coli, Lactobacillus* spp., *Micrococcus luteus* and *B. cereus;* the fungal isolates were *Aspergillus terrus, A. flavus, A. fumigatus, Fusarium oxysporum, Absidia* spp. and *Eurotium* spp. These were outlying from the different species of snails *i.e. Achatina fulica* and *Limcolaria* spp. It was deduced that snails though nutritionally rich are stockpile of pathogenic microflora which are of public health importance (Anthony *et al*. 2010).

Buruli ulcer disease is also called as common mycobacterial disease of humans. Aquatic snails act as passive hosts of *Mycobacterium ulcerans* causing chronic skin ulcers. Snails transitorily harbour *M. ulcerans* without offering favourable conditions for its growth and replication. A novel intermediate link in the transmission chain of *M. ulcerans* was with predator aquatic insects in addition to phytophage insects. Water bugs, such as *Naucoris cimicoides*, act as a potential vector of *M. ulcerans*, as they are infected specifically by this bacterium after feeding on snails having mycobacterium (Marsollier *et al*. 2004).

Water snails acts as intermediate hosts of various diseases. The digenean trematode *Nanophyetus salmincola* has a complex life cycle involving freshwater snails *Juga* spp. (Family *Pleuroceridae*) as the first intermediate hosts (Purcell *et al.* 2017). Liver fluke *Opisthorchis viverrini* causes food borne trematodiasis linking with the first intermediate snail hosts that has highly variable infection rates compared to the second intermediate fish hosts. Bithynia snails are likely to be the key link in the *O. viverrini* life cycle (Petney *et al*. 2012). Another example is the genera *Biomphalaria* and *Bulinus* that serve as intermediate hosts of digenetic trematodes of the genus Schistosoma (Morgan *et al*. 2002). According to the World Health Organization, these parasites infect 210 million people worldwide (Gryseels *et al*. 2006; Bruun and Aagaard-Hansen, 2008). The *Pomaceae canaliculata* snail is an

intermediate host of *Angiostrongylus cantonensis* the causal agent of eosinophilic meningoencephalitis in China (Deng *et al*. 2012).

Snail-borne parasitic diseases, such as schistosomiasis, angiostrongyliasis, fasciolopsiasis, fascioliasis, opisthorchiasis, paragonimiasis and clonorchiasis, create risks to human health and cause major socio-economic complications in many tropical and sub-tropical countries (Lu *et al*. 2018). According to the Nolan (2013), Swimmer's Itch is a skin inflammation suffered by humans that is caused by avian schistosomes that was transmitted by *Physid* snails. Duck was typically the definitive host in the lifecycle of an avian schistosome. Humans are exposed to parasite cercaria released from freshwater snails into the water, however, parasites do not further develop in the human host. Parasites typically die within the skin of humans, causing server itching. Swimmer's itch is formally known as cercarial dermatitis (Rao *et al*. 2007).

Numerous studies are available on the issue of compatibility, the successful or unsuccessful parasite infection on invertebrate and vertebrate hosts, especially when humans are involved in the parasite lifecycle. According to the Webster *et al*. (2012) Schistosomiasis, caused by dioecious digeneans of the genus *Schistosoma*, is a parasitic disease which have a two-host life-cycle with asexual reproduction occurring in a specific freshwater or amphibious snail and a sexual stage within mammalian host. The asexual reproduction within the obligate intermediate host snail gives rise to clonal larvae, cercariae (Brant *et al*. 2006; Colley *et al*. 2014). Snail species of the genus *Bulinus* act as intermediate hosts for several schistosome species that belong to the *Schistosome haematobium* species group in Africa and the Middle East (Rollinson *et al*. 2001; De and Wolmarans, 2005). More precisely, *B.truncatus* and *B.globosus* have been shown to transmit both *S. haematobium* and *S. bovis*, which are responsible for human urogenital and livestock intestinal schistosomiasis, respectively. There are several genera of snails acting as intermediate hosts of schistosomiasis in India (Kali, 2015).

Table 2.3 Different Genera of snails as intermediate hosts for schistosomes in India

Schistosomes species	**Intermediate hosts (snails)**
Schistosome indicum	*Indoplanorbis exustus*
S. spindalis	*I. exustus*
S. bomfordi	Unknown
S. incognitum	*I. exustus, Lymnaea luteola*
S. nasale	*I. exustus, L. luteola*
S. nairi	Unknown
Oncomelania dattai	*I. exustus, L. luteola*
O. turkestanicum	*L. auricularia*
Schistosome of Gimvi village	*Ferrissia tenuis*

Source: (Kali, 2015).

Sangwan *et al.* (*2016*) proposed that *Indoplanorbis* and *Lymnaea* are common freshwater snail species in India which act as intermediate hosts of various trematode species causing fasciolosis, amphistomosis and schistosomiasis in livestock. These diseases are important as they are widespread in India and affect livestock sector by causing substantial mortality and economic loss (Garg *et al*. 2009). The main freshwater snails found in the Haryana are *Indoplanorbis (I. exustus*) and *Lymnaea (L. luteola, L. acuminata* and *L. auricularia*) among others. The snail-borne diseases, particularly amphistomosis and fasciolosis are endemic in Haryana (Yadav *et al*. 2008).

Rekha *et al*. (2020) studied that divergence and dispensation pattern of freshwater snails and the recognisation of the schistosome contaminated snails in ten different ponds of Madurai district, Tamil Nadu, South India was perceived and collected. In the collection, six species which belongs to five families having 1250 entity of freshwater snails were found. In four collected freshwater snail species the cercariae infection was observed. Among these four infected snails, *L. luteola* had the highest proportion of cercariae infection. Conductivity, salinity and total dissolved solids of pond water take played as vital component for the spread of snails and cercariae infection.

According to Souris *et al.* (2015) fresh water apple snails play a role in the virus ecology by concentrating H_5N_1 viral particles from water and facilitating virus contact with the bird hosts that feed on them. Eaten by birds, they could be a link in the eco-epidemiological chain of bird transmission. Snails have mucus that contains Sialic acid similar to those that allow Avian Influenza Virus to bind to the cell membrane producing these acids (Burgmayr, 2006). Dutertre *et al*. (2014) reported that the defence-evoked venom of *Conus geographus* contains high levels of immobile toxins that vigrously block neuromuscular receptors, uniform with its lethal effects on humans.

Kathade *et al*. (2020) isolated yeast (*Pichia kudriavzevii*) from the intestine of edible freshwater snail (*P. globosa*), distinguish them for probiotic prospects as probiotic microorganisms has the potential to lower cholesterol levels through various mechanisms, together with the activity of bile salt hydrolase and thus, evaluate its ability to assimilate cholesterol. Snails (*Physa gyrina*) has consequences on biofilm, bacterial myriad, off-flavor-producing bacteria and off-flavor compounds in reuse aquaculture systems raising rainbow trout (*Oncorhynchus mykiss*). Eight experimental scale systems were used, including four with and without snails. Rainbow trout health and production was not ostentatious by snail presence and thus they both can co-exist in the environments (Davidson *et al.* 2019).

Kigigha and Onyema in (2015) delineated the multidrug resistant bacterial isolates from water snails and derived that *E. coli* was resistant to six out of the ten tested antibiotics viz. Gentamicin, Tetracycline, Cefuroxime, Ampicillin, Amoxicillin and Norfloxacin. While the *Staphylococcus* isolate was resistant to three tested antibiotics viz. Cephlexin, Clindamycin and Ominoxazole out of the ten.

Gauri *et al*. (2011) delved the antimicrobial peptides, a new class of antibiotics that investigated to have the activity in opposition to a wide range of bacteria susceptible to traditional antibiotics. An antimicrobial peptide of 1676 Da was refine from *Bellamya bengalensis* (fresh water snail), using ultrafiltration and reversed phase liquid chromatography. The repercussions of this peptide on *S. epidermidis* resistant to ampicillin and chloramphenicol scrutinize; peptide manifest the particularity to bacterial membranes and establish that peptide contribute in the buildout of therapeutic agent for *Staphylococcus* infections.

Essential oils of thyme, cinnamon, bay and clove have been found to be strongly antimicrobial among the many tested microbes. Essential oils (EOs) have aroused attention among the naturally-occurring bioactive agents with promising antimicrobial activity. EOs are a mixture of volatile constituents produced by aromatic plants as secondary metabolites, as a protective mechanism against predators, microorganisms or weather adversities. Among the 100,000 known secondary metabolites, EOs account for over 3000, of which about 300 have commercial interest and are used by the food, cosmetic and pharmaceutical industries (Kim *et al*.1995).

Fico *et al.* (2004) found in their respective evaluation the role of essential oils extracts at fractions, different polarity and pure compounds which was secure from *Nigella damascena* plants and seeds as it has antimicrobial and molluscicidal biological activities. Antimicrobial tests concluded that the essential oil was active only against Gram positive bacteria; among the extracts, the BuOH was active against *P. aeruginosa* and *S. aureus*. Although, molluscicidal activity was absent. Significant change in shell shape of *P. turbinatus*, with sylphlike Mesolithic shells being substituted by thickest forms in the Meso-Neolithic. These variations were spelled out with collection cart from sheltered shores in the Mesolithic to displayed rocky shores in the Meso-Neolithic, thus corroborated potential effect of human collection on size and shape of utilized species (Colonese *et al*. 2014).

According to Portet *et al.* (2019), there are only few studies that examined the pathogenic bacterial association with fresh water snail (*Biomphalaria glabrata)* in previous years. The five predominant aerobic heterotrophic bacterial genera including *Vibrio, Acinetobacter, Aeromonas, Pseudomonas* and *Enterobacter* were present in snail *(*Ducklow *et al.* 1979). Also, six additional bacteria genera i.e. *Sphingomonas, Rhizobium, Stenotrophomonas, Klebsiella, Cupriavidus* and *Citrobacter* were present in the cultured *B. glabrata* characterised using 16S rRNA sequences (Silva *et al.* 2013). Vibriosis is one of the most prevalent bacterial diseases affecting diversified shellfish and marine animals. Chong *et al.* (2011) reported that approximately 66.7% of reported diseases in groupers (*Epinephelus* spp.) is vibriosis, leading to mortality up to 50% affecting all stages of growth found by El-Galil & Mohamed (2012).

Nor-Amalina *et al.* (2017) found in recent report *V. harveyi, V. parahaemolyticus* and *V. campbelli* as the most common bacterial species infecting the farmed aquatic animals. Therefore,

several species of Vibrionaceae have been associated with health problems of aquatic animals. Oh *et al.* (2019) isolated *Staphylococcus* sp. from the *O. mykiss*. Naimi *et al.* (2003) reported zoonotic potential of *staphylococci* has led to increased interest in their transmission mechanism via food, livestock, as well as domestic and wild animals.

Defoirdt *et al.* (2011) concluded that antibiotics are used in aquaculture in attempts to control bacterial diseases. Karunasagar *et al.* (1994) reported mass mortality in *Penaeus monodon* larvae caused by *V. harveyi* strains with multiple resistance to cotrimoxazole, chloramphenicol, erythromycin and streptomycin antibiotics. Most prominent antibiotic resistant bacteria in environment are vancomycin-resistant *enterococci* (VRE), methicillin-resistant *Staphylococcus aureus (*MRSA) and multi drug-resistant *pseudomonas* (Kummerer, 2004).

Continuous and improper use of antibiotics leads to potential development of antibiotic resistant bacteria, environmental pollution and accumulation of toxic residues in aquaculture. Therefore, efforts have been made to explore natural products such as herbs in developing alternative dietary supplements that enhance growth performance and health and immune system of animals, as these products are inexpensive, safer, effective and can be easily prepared and are biodegradable. Study also included the *in-vitro* and *in-vivo* applications of herbs and plant extracts or their combinations, in relation to appetite stimulator, growth promoter, antimicrobial, antiparasitic, antioxidant and immune-stimulation in animals (Syahidah *et al.* 2015).

Plants extracts are continuing by being classified as constructive and despicable unconventional sources of medication all over the world especially in the developing countries. Kigigha and Onyema (2015) gauged the effect of cooking Egusi-soup on the antibacterial activity of bitter leaf (*Vernonia amygdalina*). The antibacterial activity of these extracts were assessed against the two pathogenic isolates viz. *S.aureus* and *E.coli* from hospital origin. Crude extracts of curcuminoids and essential oil of *Curcuma longa* varieties (*Kasur*, *Faisalabad* and *Bannu*) were considered for their antibacterial activity against four bacterial strains viz., *B. subtilis, B. licheniformis, B. macerans* and *Azotobacter* using agar well diffusion method. Both Curcuminoids and oil manifest the zone of inhibition against all tested strains of bacteria. Among all the bacterial strains *B. subtilis* was the most tactful to turmeric extracts of curcuminoids and oil (Naz *et al*. 2010).

Vaseeharan and Thaya (2014) reported that the immunostimulants are considered as an alternative for antibiotics, which will boost the immune system of the cultured organism, thus effectively countering the assault of pathogens. The use of plant materials as immunostimulant will be an eco-friendly approach for the control of pathogens. Maqsood *et al*. (2011) reported the immunostimulant activity of glucan, chitosan, levamisole and herbal plant products such as from *Eclipta erecta, Hygrophila spinosa, Ocimum sanctum, Withania somnifera, Zingiber officinale, Andrographis paniculata, Solanum trilobatum, Psoralea corylifolia* and *Picrorhiza kurroa* having

characteristics of growth promotion, anti-stress, immune stimulation and anti-bacterial activity. Some natural compounds species such as lemon balm (*Melissa officinalis),* garlic (*Allium sativum*) and tee tree (*Melaleuca alternifolia*) act as broad-spectrum antimicrobial agents (Heinrich *et al.* 2004).

Indiscriminate and careless use of antibiotics leads to the development of antibiotic resistance in microorganisms, which is now a major health concern worldwide (Karim *et al*. 2018). Therefore, herbal extracts could be used as safe and alternative to synthetic antibiotics for the management of bacterial diseases (Hannan *et al.* 2019). According to Moussa *et al.* (2012) well-known natural product-honey has *in vitro* antibacterial activity against *S. aureus* and *Streptococcus pyrogenes*. Also, *Juglans regia* (walnut) in combination with *Camellia sinensis* (tea plant, tea shrub) acts synergistically with different antimicrobial agents to inhibit multiple-resistance bacteria (MDR), most likely targeting the bacterial cell wall (Farooqui *et al.* 2015).

CHAPTER-III

MATERIALS AND METHODS

The present investigation entitled "**Efficacy of essential oils against bacterial isolates from fresh water snails"** deals with the information on bacterial diversity of fresh water snails in selected areas of the district Hisar of the state Haryana. The four different sites in and around district Hisar (Haryana) including lotus pond of the Chaudhary Charan Singh Haryana Agricultural University, Hisar (29°08'59.1"N 75°42'16.8"E) were chosen for this study.

Haryana attained statehood from Panjab on 1st November 1966. Haryana is a major state of Northern India. Haryana shares its borders with Delhi in the South East, Uttar Pradesh and Uttarakhand in the East, Punjab in the West, Chandigarh and Himachal Pradesh in the North and the great spread of Rajasthan in the South. *Shivalik* hills in the North, *Aravalli* hills in the West and South are the major hills of the state. Its geographical position is between 27.39° N and 30.35° N latitude & between 74.28° and 77.36° longitude. The altitude of Haryana varies between 700 to 3600 feet (200 meters to 1200 meters) above sea level. The total area of the state of Haryana is 44,212 square kilometers. Out of this, 1,553 square kilo-meters of area is covered by forests (ICFA, 2016).

Hisar district is one of the 22 districts of Haryana in north-western India. It serves as district headquarters. Hisar is located at 29.09°N 75.43°E in western Haryana. It has an average elevation of 215 m (705 ft) above mean sea level. It is located 164 km (102 miles) to the west of New Delhi, India's capital, and has been identified as a counter-magnet city for the National Capital Region to develop as an alternative center of growth to Delhi.

Hisar has a continental climate, with very hot summers and relatively cool winters. The main characteristics of climate in Hisar are dryness, extremes of temperature and scanty rainfall. The average temperature in Hisar is 25.1 °C. About 459 mm of precipitation falls annually.

Technical programme of the study was carried out in two phases. The first phase covered to isolate and characterize the bacterial isolates retrieved from fresh water snails. The second phase was concerned with the evaluation of antibacterial activity of various antibiotics and essential oils against bacterial isolates.

Objective :1 To isolate and characterize the bacterial isolates retrieved from fresh water snails

a) The study was carried out in Rodentology laboratory of the Zoology Department, CCS Haryana Agricultural University, Hisar (Haryana). The surveys were conducted for collection of fresh water snails from nearby village ponds of district Hisar following standard protocols (Brown, 2002). Fresh

water snail samples were collected from different selected sites. Lab experiments were conducted under standard conditions (Bartlett and Trust, 1976).

b) Photographs taken at different stages of the study are depicted in Chapter "Results".

Materials used and methodology adopted for different experiments under laboratory and in collection of sampling are outlined below under relevant sub-headings.

3.1) Materials

Materials used during study period are: sterile glass vials, polythene bag, protective gloves, slides, petri plates, immersion oil, nutrient agar, specific media, autoclave, compound microscope, laminar air flow and B.O.D. incubator etc.

Objective: (1) To isolate and characterize the bacterial isolates retrieved from fresh water snails

a) The samples were collected from visceral tissues/mass collected from snails after surface sterilization in sterile glass test tubes.

b) The bacterial cultures were isolated from retrieved samples using pour plate dilution technique on Nutrient agar (NA) medium.

c) Bacterial colonies of different morphology were picked up, purified and maintained on Nutrient/ EMB/Mac-Conkey/LB agar slants for further studies.

d) The different bacterial isolates were characterized on the basis of standard morphological and biochemical tests for their identification (Quinn *et al.* 1994).

Photographs taken at different stages of experiments are shown in Chapter "Results". Materials used and methodology adopted for different experiments under laboratory and during sampling are outlined below under relevant sub-headings.

3.1.1 Methodology:

❖ **Collection of samples**

The samples were collected from visceral tissues/mass collected from snails after surface sterilization in sterile glass vials which was filled one third with nutrient broth. The study targeted was conducted at Rodentology laboratory in the Department of Zoology, CCS Haryana Agricultural University.

❖ **Culturing of bacteria using Dilution plate technique**

i. In dilution plate technique, 1ml of sample from glass vail was thoroughly mixed in 9ml of sterile water. Then, 0.1ml of this solution was pipetted out in 0.9ml dilution blank in Eppendrof ™ tube aseptically. Contents of tubes were shaken well on shaker. Using a fresh pipette 0.1ml of diluted culture was transferred into another 0.9ml dilution blank. The process was repeated till 10^{-5} dilution.

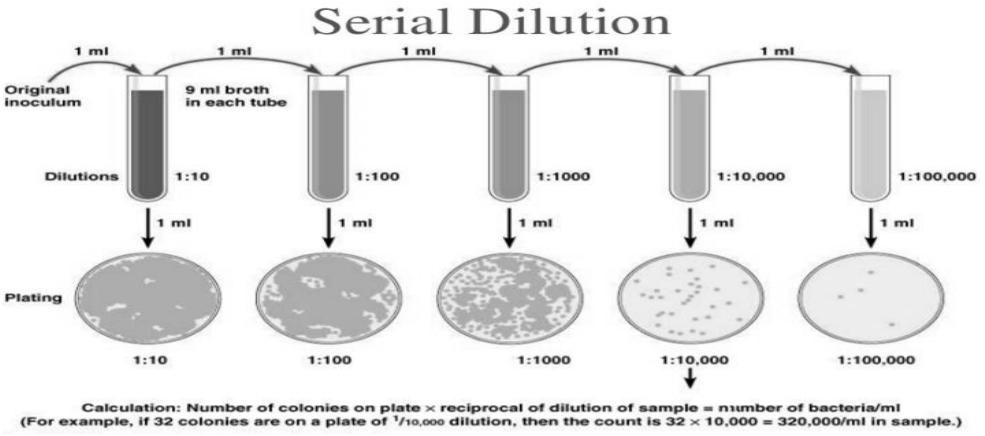


Figure 1: Serial dilution technique (Source: Pearson, 2004)

ii. From the dilution of 10^{-4} of sample, 0.1ml aliquot was transferred aseptically onto freshly prepared nutrient agar plates. It was spread on the plate with a sterile bent glass rod.

iii. The inoculated plates were inverted and incubated at 37 ± 2^0C for 24 hours in BOD. Then, the plates were examined for growth.

iv. Pure cultures of bacteria were obtained by aseptically streaking representative colonies of different morphological types (circular, irregular, rhizoid), which appear on the cultured plates onto freshly prepared nutrient agar plates from the incubator.

v. Discrete bacterial colonies of different morphological characteristics like colony appearance, margin of colony (entire, serrate, lobate, undulate, filamentous) and elevation (flat, raised, convex, umbonate) were developed and were sub-cultured on nutrient agar slants and incubated at 37 ± 2^oC for 24hrs.

vi. These served as pure stock cultures for subsequent Gram staining test, biochemical characterization tests and oxidation-fermentation tests (Brown, 2005).

❖ **Identification of bacteria**

Isolated pure cultures of bacteria were subjected to a number of important biochemical tests (primary and secondary tests for identification) reported by Kreig & Holt (1984) "Bergey's Manual of Determinative Bacteriology" and Quinn *et al.* (1994).

a) Primary identification

The pure bacterial colonies were subjected to following tests, which gave primary identification of the bacterium to the generic level.

❖ **Gram staining**

The Gram staining was done following the method of Hans Christian Gram. The bacteria which showed the purple/violet colour was classified as Gram positive and which showed the pink colour was identified as Gram negative.

❖ **Reagents:**

Crystal violet, Gram's iodine solution, Acetone/ Ethanol, Safranin and distilled water.

❖ **Procedure:**

1. Bacterial slide smear preparation

A drop of water was taken on the slide and aseptically transferred a minute amount of bacterial pure colony on it with the help of sterilized inoculation loop. The culture was spread with the help of inoculation loop to an even thin film over a circle of 1.5 cm in diameter. Material was fixed on a slide with methanol or heat. If the slide was heat fixed, allowed it to cool to the touch before applying the stain.

2. Gram Staining Procedure/Protocol:

a) Heat-fixed smear of bacterial colony was flood air-dried, for 1 minute with crystal violet staining reagent.

b) Slide was washed in a gentle and indirect stream of tap water for 2 seconds.

c) Slide was flooded with the mordant: Gram's iodine and wait 1 minute.

d) Slide was washed in a gentle and indirect stream of tap water for 2 seconds.

e) Decolorizing agent (Acetone-alcohol decolorizer) was flooded on the slide for 10-15 seconds or added drop by drop to slide until decolorizing agent running from the slide runs clear.

f) Slide was flooded with a counterstain, safranin. Wait 30 seconds to 1 minute.

g) Slide was washed in a gentle and indirect stream of tap water until no colour was appeared in the effluent and then blotted with absorbent paper to remove the excess water.

h) Results of the staining procedure were observed under oil immersion (100x) using a Bright field microscope (Carl Ziess Trinocular™).

3. Gram reaction- Gram stain smear was prepared from a pure culture for the recording the Gram reaction as Gram- positive or Gram- negative and the cellular morphology (coccus, rod or bacillus).

❖ **Catalase test**- The catalase enzyme reaction is detected by this test. This reaction converted hydrogen peroxide into water and gaseous oxygen. A clean glass slide was taken and the bacterial cells were placed on it. A drop of 3% hydrogen peroxide was added on it. A positive reaction is confirmed if within a few second, effervescence of oxygen gas occurs.

❖ **Oxidase test**- The presence of cytochrome-c oxidase in a bacterial cell is confirmed by this test. Anaerobic bacteria were oxidase negative. Initially, the nutrient broth was prepared. Then, the bacterial cultures were poured into the nutrient broth test tube with the help of sterilized loop and incubated in BOD for 24 h for growth. Growth was observed in the test tube and oxidase disc was

poured into test tube with the help of sterilized forceps. Appearance of blue colour after 30-40 sec, confirmed the positive reaction.

b) **Secondary characterization-** Once the bacterium was identified to a generic level, further tests were carried out to identify the species of the particular bacteria.

1) Malonate broth

Malonate (0.3% Sodium malonate) is utilized by many bacteria as sole source of carbon. The indicator used was Bromothymol blue. Bromothymol blue was green at neutral pH in an uninoculated tube and changed to deep blue, which showed the positive test and no change in colour showed the negative test. Finally, the bacterial culture tubes were incubated at 37±2°C for 24-48 h in BOD.

2) Urea broth base agar

This medium is used for the differentiation of *Proteus* species from *Salmonella* and *Shigella* species, based on urea utilization. Gram-negative enteric bacilli are unable to utilize urea because of less nutrients and high buffering capacity of the medium. Urea Broth becomes alkaline as the utilization of urea by the organisms liberates ammonia during the incubation, indicated by pink red colour. All urea test media rely on the alkalinity formation and so they are not specific for urease testing. The utilization of proteins may raise the pH to alkalinity due to protein hydrolysis and excess of amino acids results in false positive reaction. A medium without urea serves as negative control to rule out false positive results. A deep pink colour at the top of the tube in the medium within 8 h indicated the prompt hydrolysis of urea. This colour spread throughout the whole culture tube within 18 h.

3) Pseudomonas agar F Base

The isolation and differentiation of *Pseudomonas* sp. was done by using this selective culture media. It was based on the formation of pyocyanin and/or pyorubin or fluorescein. *Pseudomonas* Agar P favored the formation of pyocyanin and/or pyorubin and reduced the formation of fluorescein, whereas *Pseudomonas* Agar F stimulated the production of fluorescein and reduced pyocyanin and/or pyorubin production. The culture plates were incubated at 37±2°C up to 24 hrs.

4) LB Agar (Luria Bertani agar)

The prepared media was stored below 8°C and protected from direct light. The bacterial plates were incubated at 37±2°C for 24 h.

5) Nutrient agar (NA)

This culture medium was used for less fastidious microorganisms as well as for permanent culture. The cultured plates were incubated for at 37±2°C for 24 h.

6) Violet Red Bile Glucose Agar

The isolation, detection and enumeration of coli-aerogenes bacteria were done by using Violet Red Bile Glucose Agar. The coliform group belongs to the family Enterobacteriaceae. It consists of several genera of bacteria. The historical definition of this group has been based on the method used for detection i.e. lactose fermentation. This group includes aerobic and facultative anaerobic, Gram-negative, non-spore-forming rod shape bacteria that ferment lactose within 48 hours at 35°C with gas and acid formation. The growth of many unwanted organisms is suppressed due to selective inhibitory components such as crystals violet and bile salts while tentative identification of sought bacteria can be made by the indicator lactose system and neutral red of the Violet Red Bile Glucose Agar. Organisms, which rapidly attack lactose, produce purple colonies surrounded by purple halos and non-fermenters or late lactose-fermenters produce pale colonies with greenish zones.

7) Brilliant Green Agar Base, Modified

Selective isolation of Salmonellae other than *Salmonella typhi* from faeces and other materials was done by using Brilliant Green Agar (Modified). Brilliant green of this medium inhibits growth of majority of Gram-negative and Gram-positive bacteria. Most affected organisms are *S. typhi*, *Shigella* species, *E. coli*, *Pseudomonas* species and *S. aureus*.

8) Endo agar

This test was done for the selective isolation, cultivation and differentiation of coliform and other enteric bacteria based on their ability to ferment lactose. Lactose fermenters appeared as dark red colonies with a gold metallic sheet and non-lactose fermenters appeared as colourless or translucent colonies. The cultures plates were incubated at 37±2°C for 24 h aerobically.

9) Cetrimide agar base

A modification of the medium was proposed by Brown and Lowbury (1965) for the isolation and differentiation of *Pseudomonas aeruginosa* from others Gram-negative non-fermentative bacterium. This compound largely inhibited the growth of the accompanying microbial flora. The pigment production of *P. aeruginosa* was not inhibited on this medium. The bacterial plates were incubated for 48h. at 37±2°C aerobically.

10) MacConkey agar

MacConkey agar was widely used as selective and differential culture medium for Gram-negative bacteria, which inhibited the growth of Gram-positive bacteria. In addition to the nutrient agar, base of bile salts and crystals violet inhibits the growth of Gram-positive bacteria and makes the MacConkey agar selective. Lactose (fermentable carbohydrate) and neutral red pH indicator were added to differentiate the lactose positive coliforms from the potentially pathogenic lactose non- fermenters. When the lactose is fermented, acid products lowered the pH below 6.8, which resulted the bacterial colonies into pinkish-red in colour. If an organism was unable to ferment lactose, the colonies appeared colourless. The culture plates were incubated at 37±2°C for 24 h aerobically.

MacConkey agar selective: Gram-negative organisms grow, Gram positive organisms will not grow due to addition of bile salts and crystals violet.

MacConkey agar differential: Lactose fermenters appeared pink, non- lactose fermenters appeared colourless. This was due to addition of indicator, neutral red.

11) Eosin-Methylene blue (EMB) agar

This agar was a selective and differential medium was used for the isolation and differentiation among members of the Enterobacteriaceae. Eosin methylene blue agar (EMB) selected the Gram-negative bacteria, and differentiates among Lactose fermenters and non- fermenters. EMB agar contained methylene blue and eosin dyes to inhibit the growth of Gram-positive bacteria. Small amounts of acid production resulted in a pink colour growth, while large amounts of acids caused the acid to precipitate on the colony, resulted in a characteristic greenish, metallic sheen. Organisms, which do not ferment lactose, remained colourless. The culture plates were incubated at 37±2°C for 24-48 h aerobically.

12) Voges-Proskauer (VP) test

The organisms produce acetoin from the degraded glucose during 2,3-butanediol fermentation. In Voges-Proskauer test, VP broth was used. The chief end products of glucose metabolism were acetoin and 2,3-butanediol. After 48 h of incubation, Barritt's Reagent-A (alpha-naphthol) and Barritt's Reagent- B (potassium hydroxide) were added to the sample and the tubes were gently shaken for aeration, formation of a red colour indicated the negative results.

13) Muller -Hinton agar

Muller-Hinton agar is a microbial growth medium that is commonly used for antibiotic susceptibility testing. It is a non-selective, non-differential medium. This means that almost all organism plated on here will grow, Additionally, it contains starch. Starch is known to absorb toxins released from the bacteria, so that they cannot interfere with the antibiotics. Second, it is a loose agar. This allows for better diffusion of the antibiotics than other plates. Better diffusion leads to a truer zone of inhibition.

14) SS Agar (*Salmonella-Shigella* Agar)

SS Agar medium is recommended as differential and selective medium for the isolation of *Salmonella* and *Shigella* species from pathological specimens. SS Agar is a moderately selective medium in which Gram-positive bacteria are inhibited by bile salts, brilliant green and sodium citrate. Lactose is the fermentable carbohydrate. Brilliant green, bile salts and thiosulphate selectively inhibit Gram-positive and coliform organisms. Sodium thiosulphate is reduced by certain species of enteric organisms to sulphite and H_2S gas and this reductive enzyme process is attributed by thiosulphate reductase. Production of H_2S gas is detected as an insoluble black precipitate of ferrous sulphide, formed

upon reaction of H_2S with ferric ions or ferric citrate, indicated in the center of the colonies. Lactose fermenters appeared pink, non- lactose fermenters appeared colourless with or without black centre. This was due to addition of indicator- neutral red. Growth of *Salmonella* species appears as colourless colonies with black center resulting from H_2S production. *Shigella* species also grow as colourless colonies which do not produce H_2S.

15) Thiosulfate-Citrate-Bile salts -Sucrose agar (TCBS) Agar

TCBS agar used for the selective isolation and cultivation of *Vibrio cholerae* and other enteropathogenic *Vibrios* species. Proteose peptone and yeast extract provide nitrogenous compounds, vitamin B complex and other essential growth nutrients. Bile, a derivative of bile salts and sodium citrate inhibit Gram-positive bacteria and coliforms. Sodium thiosulphate serves as a good source of sulphur, which in combination with ferric citrate detects the production of hydrogen sulphide. For the metabolism of Vibrio's, sucrose is added as a fermentable carbohydrate. *Vibrio* that is able to utilize sucrose will from yellow colonies. Bromothymol blue and thymol blue are the pH indicators. The alkaline pH of the medium improves the recovery of *V. cholerae*. Strains of *V. cholerae* produce yellow colonies on TCBS Agar because of fermentation of sucrose. *V. alginolyticus* also produce yellow colonies. *V. parahaemolyticus* is a sucrose non-fermenting organism and therefore produces blue-green colonies, as does *V. vulnificus.*

16) Blood Agar Base No. 2

Blood Agar Base No. 2 is a highly nutritive medium. Microorganisms producing haemolysin give visible haemolytic zones on this medium. It also serves as a differential medium for *Brucella* and *Campylobacter* species by adding different antibiotic supplements for the respective bacteria. Supplementation with sheep blood (5-10%) provides additional growth factors and also serves as basis for determining haemolytic reactions. Haemolytic patterns may vary with the source of animal blood or type of base medium used.

17) M-Enterococcus Agar Base

M-*Enterococcus* Agar is used for the detection of *Streptococcus* and *Enterococcus* groups using the membrane filtration technique.

Sodium azide inhibits Gram-negative organisms. Triphenyl Tetrazolium Chloride serves as a rapid indicator of bacterial growth. TTC is reduced to insoluble formazan inside the bacterial cells, which gives red coloration to colonies. The medium was inoculated by streaking the specimen and incubating the plates at 35-37°C for 24-48 hours. After incubation, count all light and dark red colonies as *Enterococci*.

18) Vogel- Johnson Agar Base w/o Tellurite (V.J. Agar)

V.J. agar is a selective medium for the detection of coagulase positive, mannitol fermenting *S. aureus*. Selection and differentiation of coagulase-positive *staphylococci* on V.J. Agar is based on mannitol fermentation and tellurite reduction. During the first 24 hours, contaminating organisms are almost inhibited

by tellurite, lithium chloride and high glycine content. The effect of inhibitors on *S. aureus* is reduced because of the presence of mannitol and glycine. Coagulase-positive *staphylococci* reduce potassium tellurite to metallic free tellurium and thus produce black colonies surrounded by yellow zones. This yellow colour is due to phenol red indicator that turns yellow in acidic condition due to mannitol fermentation. If mannitol is not fermented, yellow zones are not formed. Also, the colour of the medium around the colonies may even be a deeper red than normal due to utilization of the peptones in the medium. Prolonged incubation may result in growth of black coagulase-negative colonies.

19) *Aeromonas* Isolation Medium Base:

Recommended for selective and differential isolation of *A. hydrophila* from clinical and environmental specimens. The selectivity of the medium is increased by the addition of Ampicillin (FD039). Peptone special and yeast extract provide essential nitrogenous and carbonaceous compounds, long chain amino acids, vitamins and other essential growth nutrients. The salts provide the essential minerals and electrolytes. Sodium chloride maintains osmotic equilibrium. Lactose, sorbose, inositol and xylose are sources of carbon and energy. Ampicillin, bile salts and sodium thioglycollate makes the medium selective. Bromothymol blue and thymol blue acts as indicators giving the characteristic colony colour.

Objective: (2) To Evaluate the antimicrobial activity of essential oils against bacterial isolates

3.2.1 Test of essential oils (EOs)

The essential oils used in this study (i.e. Citronella, Lemon, Eucalyptus) were purchased from the local market (Research fine lab chem industries™, Mumbai). Dilutions of the EOs, for agar disc diffusion assay, were made with dimethyl sulfoxide (DMSO). The dilutions, in final volume of 2ml were 100%, 50%, 25% (vol/vol) and control. The inoculums were prepared by growing the various bacterial species on separate agar plates and colonies from the master plate were transferred with inoculating loop. Every plate was marked and divided into four parts viz. 100%, 50%, 25% and control with the help of marker. Then the bacterial culture was spread on NA plates with the help of spreader. With the help of a sterile pipe holder made wells in the agar plates. The 1 µl of each EO oil's dilution and placed in the well of the inoculated agar. The plates were incubated at 37±2°C for 24 hrs. aerobically. After incubation, the diameter of the inhibition zones was measured in centimeters from the center of hole.

The strain sensitivity to each EO dilution was classified by the measured diameter of the inhibition zones. The addition of DMSO, an aprotic organic solvent belonging to the category of sulfoxides, had the purpose of facilitating the solubilization of EOs in the culture media. EOs dilutions were prepared just before the experiments. All EOs were stored at 4° C in darkness before use and utilized before the expiration date.

Plate 1: Fresh water snail (Water canal, near Satrod)

Plate 2: Collected fresh water snails (water canal, near Satrod and Azad nagar)

Plate 3: Fresh water snail (water canal, near Balsamand road)

Plate 4: Enlarge view of fresh water snail (water canal, near Balsamand road)

Plate 5: Fresh water snail (rice fields, Fatehabad)

Plate 6: Collected fresh water snail (rice fields, Fatehabad)

Plate 7: Fresh water snail in lotus pond, CCS HAU, Hisar

Plate 8: Enlarge view of snails (lotus pond, CCS HAU, Hisar)

Reported molluscan fauna in the study area

Plate 9: VRBG + Aeromonas agar + Blood agar + Malonate Agar

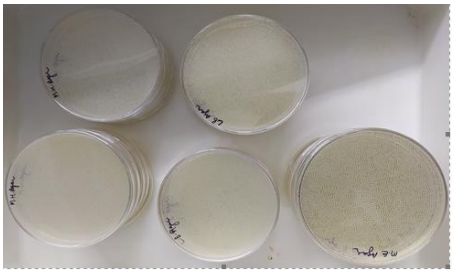


Plate 10: Mueller-Hinton & Luria Bertani Agar

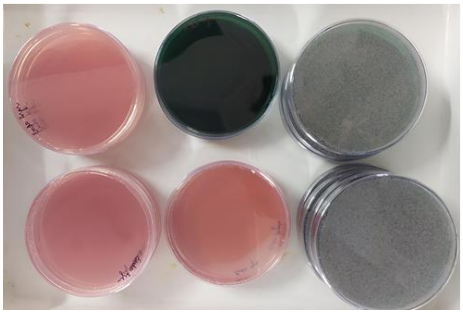


Plate 11: Endo & TCBS Agar

Plate 12: M-Enterococcus Agar

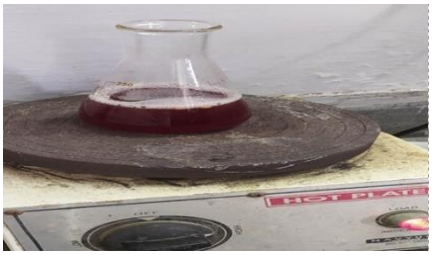


Plate 13: *Salmonella Shigella* Agar

Plate 14: Agar plates placed in B.O.D.

Differential medium agar plates

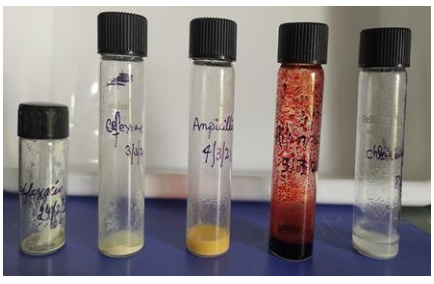


Plate 15: Antibiotics used in study

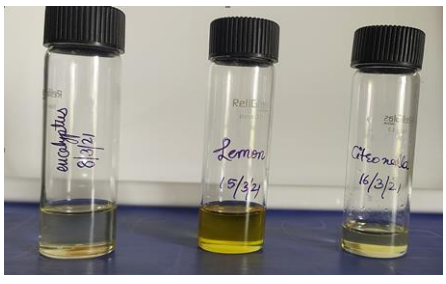


Plate 16: Essential oils (EOs) used in study

3.2.2 To test the susceptibility of different antibiotic against bacterial isolates

To test the susceptibility of the different bacterial isolates against different antibiotics, commercially

available tablets of respected antibiotics were purchased and used. Dilutions were prepared from antibiotic tablets as 100 %, 50% and 25% with the help of distilled water dilutions were prepared, which is also used as control. The inoculums were prepared by growing the various bacterial species on separate master agar plates and colonies from the plate were transferred with inoculating loop into fresh prepared NA plates. Every plate was marked and divided into four parts *i.e.* 100%, 50%, 25% and control with the help of marker. The bacterial isolates were transferred to NA plates with the inoculation loop and spreaded with the help of glass spreader. Holes were made with the help of metallic holer and these were filled with 1 µl of antibiotic's each dilution with respected demarcation. The plates were incubated overnight at 37±2°C. After incubation, the zone of inhibition was measured from the center of hole and recorded.

We applied the four different concentrations of five antibiotics viz. Cefexime, Ampicillin, Chloramphenicol, Ofloxacin and Rifampin + Isoniazid on all inoculated agar separately and the plates were incubated overnight at 37±2°C. The zone of inhibition was measured and recorded.

3.3 Statistical Analysis (Student T-Test)

Student T-test was applied for compared the efficacy of each essential oils, efficacy of each essential oils with the bacterial isolates and cumulative efficacy of all essential oils with all the bacterial isolates. We compared the efficacy of each antibiotic with all essential oils and observed the Null hypothesis for better efficacy of essential oils.

CHAPTER-IV

RESULTS

The results of the present investigation entitled "**Efficacy of essential oils against bacterial isolates from fresh water snails"** are presented under the relevant headings in this chapter. The study was carried out in Rodentology laboratory, Department of Zoology & Aquaculture, COBS&H, CCS Haryana Agricultural University, Hisar (Haryana) (29°08′25.5"N 75°40′55.5"E) and nearby villages of district Hisar. Results are interpreted and presented with the help of photographs, figures and tables.

4.1 Place of samples

The samples were collected from district Hisar and near around districts.

Table 4.1: Geographical location of sample collection sites

Sr. No. (Site no.)	**1**	**2**	**3**	**4**	**5**
Sample collection site	**Water canal (near Satrod)**	**Water canal (Azad nagar, Hisar)**	**Water canal (Balsamand road, Hisar)**	**Rice fields (Fatehabad)**	**Lotus pond (near Nehru library, CCS HAU, Hisar)**
Geographical location	29°07′10.4"N 75°47′42.1"E	29°07′46.9"N 75°42′49.6"E	29°09′49.7"N 75°41′26.9"E	29°35′39.6"N 75°29′6.2"E	29°08'59.1"N 75°42'16. 8"E

4.2 To isolate and characterize the bacterial isolates retrieved from fresh water snails

The studies were carried out in Rodentology laboratory, Department of Zoology & Aquaculture, COBS&H, CCS Haryana Agricultural University, Hisar (Haryana).

4.2.1 Isolation of bacteria

The samples were collected from visceral tissues/mass collected from snails after surface sterilization in sterile glass test tubes. Later, the bacterial cultures were isolated using pour plate dilution technique on Nutrient agar medium. Isolated bacteria are depicted in plates & tables.

Plate 17: **Water canal (near Satrod)**

Plate 18: Water canal (Azad nagar, Hisar)

Plate 19: Water canal (Balsamand road, Hisar)

Plate 20: Rice fields (Fatehabad)

Plate 21: Lotus pond (near Nehru library, CCS HAU,Hisar)

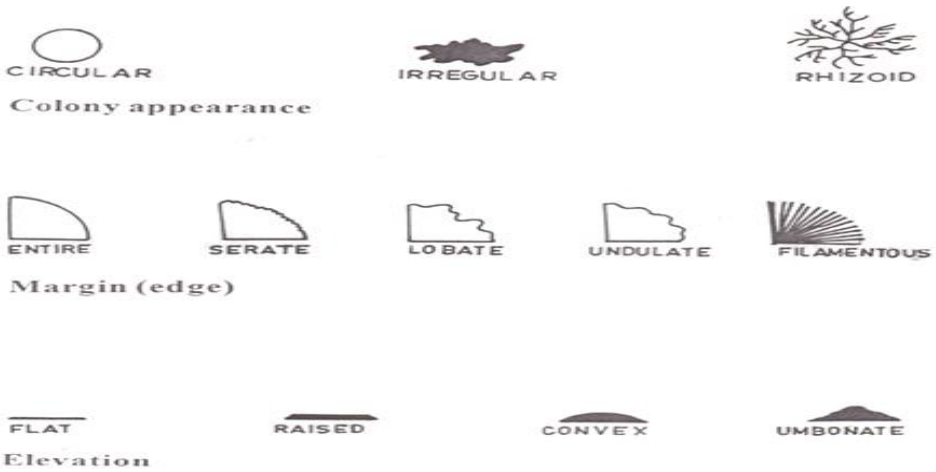


Figure 2: Colony morphology of the bacteria (Garg & Dogra, 1997)

Table 4.2: Nomenclature of bacteria isolated from collected samples					
Sr. No.	**1**	**2**	**3**	**4**	**5**
Source of snail	**Water canal (near Satrod)**	**Water canal (Azad nagar, Hisar)**	**Water canal (Balsamand road, Hisar)**	**Rice fields (Fatehabad)**	**Lotus pond (CCS HAU, Hisar)**
Nomenclature of isolates	HSa, HSb, HSx, HSj	HAd, HAo, HAn, HAf	HB1, HB2, HB3, HB4, HB5, HB6. HB7, HB8, HB9, HB10, HB11, HB12	FR1, FR2, FR3, FR4, FR5, FR6. FR7, FR8,	HP1, HP2, HP3, HP4, HP5, HP6. HP7, HP8, HP9, HP10

The morphological characteristics i.e. colony appearance, margin, elevation, Gram staining and shape of pure bacterial isolates were observed and recorded (table 4.3). Most of the bacterial colonies were found circular, shiny, entire and convex/ raised (figure 2) except HB1, HB8, HB12, FR4 and HP9 were irregular, lobate and flat and HP3 was irregular, ungulate and umbonate. Mostly bacterial isolates were Gram negative and some are Gram positive. The bacterial isolates were rod or cocci shaped.

Table 4.3: Morphological characteristics of bacterial isolates on nutrient agar medium						
Sr. no.	**Character**	**Colony appearance**	**Margin**	**Elevation**	**Gram's Test**	**Shape**
1.	**HSa**	Circular	Entire	Convex	+	Cocci
2.	**HSb**	Circular	Entire	Convex	-	Cocci
3.	**HSx**	Circular	Entire	Convex	-	Cocci
4.	**HSj**	Circular	Entire	Convex	-	Rod
5.	**HAd**	Circular	Entire	Convex	+	Cocci
6.	**HAo**	Circular	Entire	Convex	+	Cocci
7.	**HAn**	Circular	Entire	Convex	+	Rod
8.	**HAf**	Circular Shiny	Entire	Convex	-	Rod
9.	**HB1**	Irregular	Lobate	Flat	-	Cocci
10.	**HB2**	Circular	Entire	Raised	-	Cocci
11.	**HB3**	Circular	Entire	Convex	+	Cocci
12.	**HB4**	Circular	Entire	Convex	-	Rod
13.	**HB5**	Circular	Entire	Convex	+	Cocci
14.	**HB6**	Circular	Entire	Flat	+	Cocci
15.	**HB7**	Circular Shiny	Entire	Convex	-	Rod
16.	**HB8**	Irregular	Entire	Flat	+	Cocci
17.	**HB9**	Circular	Entire	Convex	+	Rod
18.	**HB10**	Circular	Entire	Raised	-	Cocci
19.	**HB11**	Circular	Entire	Convex	+	Cocci
20.	**HB12**	Irregular	Lobate	Flat	-	Cocci
21.	**FR1**	Circular	Entire	Convex	+	Rod
22.	**FR2**	Circular	Entire	Convex	+	Cocci
23.	**FR3**	Circular	Entire	Convex	-	Rod
24.	**FR4**	Irregular	Lobate	Flat	+	Rod
25.	**FR5**	Circular	Entire	Convex	+	Rod
26.	**FR6**	Circular	Entire	Convex	+	Cocci
27.	**FR7**	Circular Shiny	Entire	Convex	-	Rod
28.	**FR8**	Circular	Entire	Convex	+	Rod
29.	**HP1**	Circular	Entire	Raised	-	Cocci
30.	**HP2**	Circular	Entire	Raised	-	Cocci
31.	**HP3**	Irregular	Ungulate	Umbonate	-	Rod
32.	**HP4**	Circular	Entire	Convex	-	Rod
33.	**HP5**	Circular	Entire	Raised	-	Cocci
34.	**HP6**	Circular	Entire	Raised	-	Cocci
35.	**HP7**	Circular Shiny	Entire	Convex	-	Rod
36.	**HP8**	Irregular	Filamentous	Flat	+	Rod
37.	**HP9**	Irregular	Lobate	Flat	+	Rod
38.	**HP10**	Circular	Entire	Convex	-	Rod

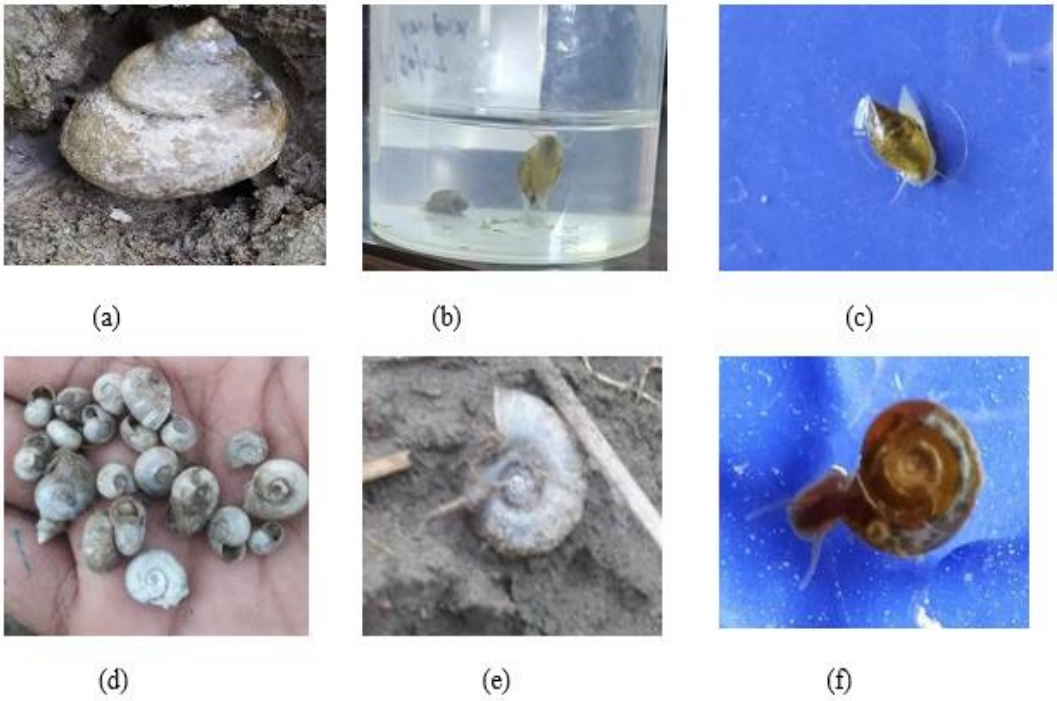


Plate 22: Fresh water snails collected from different geographical sites

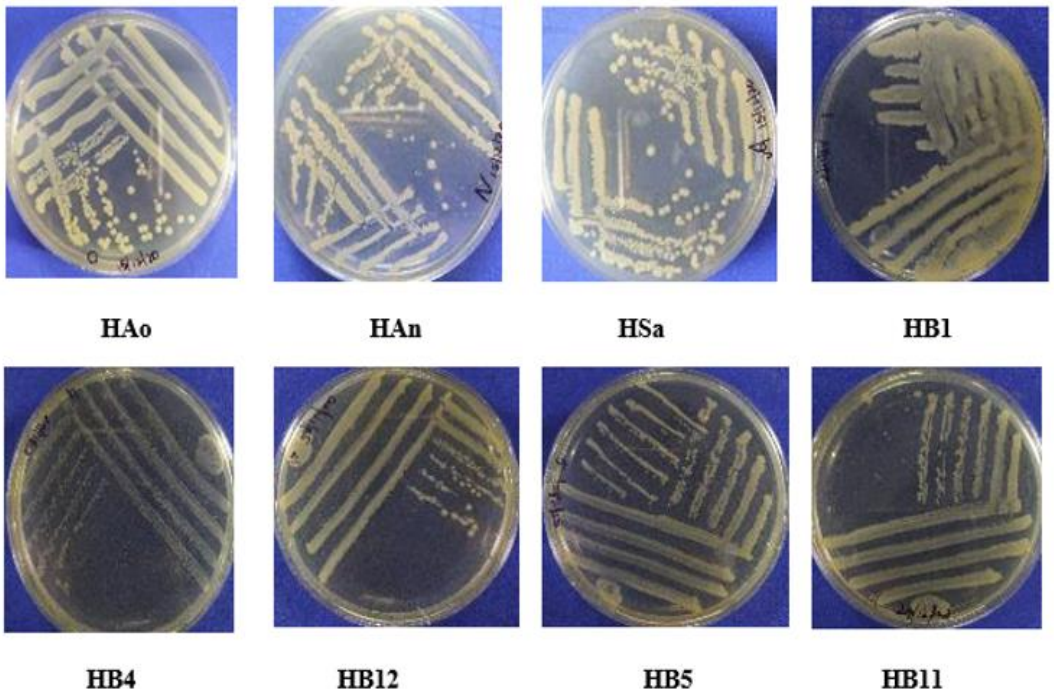


Plate 23.a: Various bacterial isolates on the Nutrient Agar

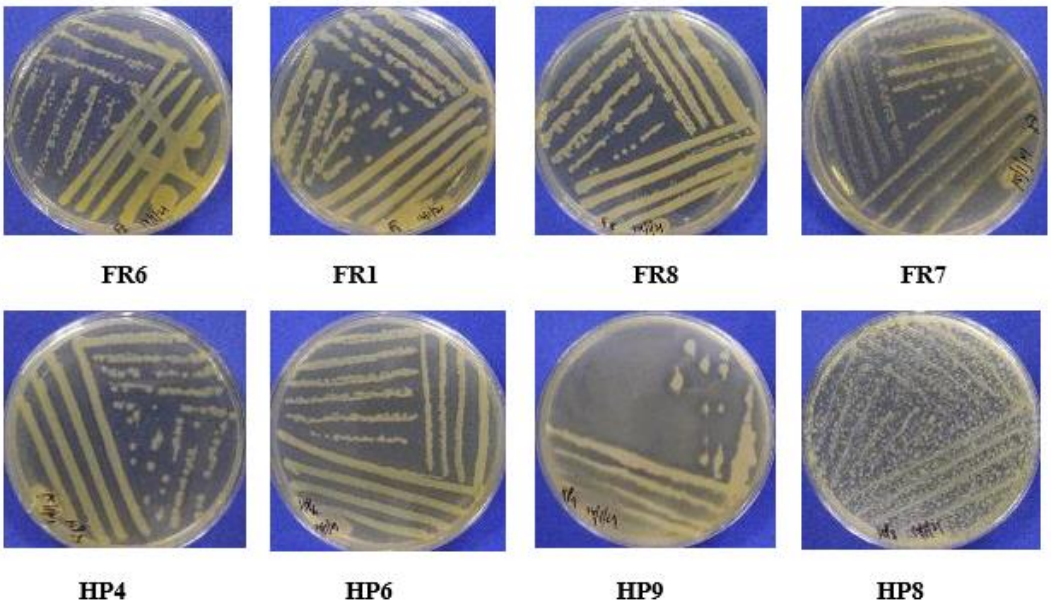


Plate 23.b: Various bacterial isolates on the Nutrient Agar

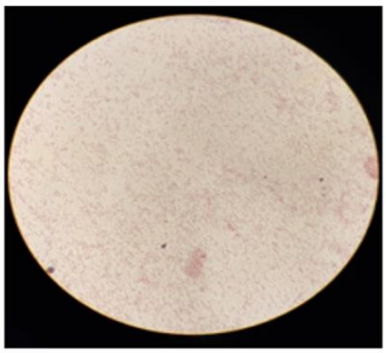


Plate 24: Gram negative bacterial slide

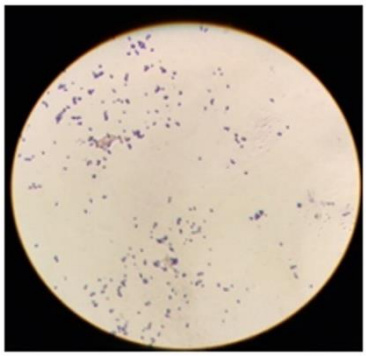


Plate 25: Gram positive bacterial slide

Plate 26: Catalase test

Plate 27: Oxidase test

4.2.2 Biochemical characterization of isolated bacteria from fresh water snail

4.2.2.1 Primary biochemical test

After obtaining the pure culture of bacteria isolates, they were subjected to primary biochemical test i.e. catalase and oxidase test (Himedia™ chemicals) using standard protocols and results were recorded (Table 4.4 and Plates 24-27).

Table 4.4: Primary biochemical test for bacteria isolated from fresh water snails			
Sr. no.	**Character**	**Catalase test**	**Oxidase test**
1.	**HSa**	+	-
2.	**HSb**	+	+
3.	**HSx**	+	+
4.	**HSj**	+	-
5.	**HAd**	+	+
6.	**HAo**	+	-
7.	**HAn**	+	-
8.	**HAf**	+	-
9.	**HB1**	+	+
10.	**HB2**	+	+
11.	**HB3**	+	+
12.	**HB4**	+	-
13.	**HB5**	+	+
14.	**HB6**	-	-
15.	**HB7**	+	-
16.	**HB8**	-	-
17.	**HB9**	+	-
18.	**HB10**	+	+
19.	**HB11**	+	+
20.	**HB12**	+	+
21.	**FR1**	+	-
22.	**FR2**	+	-
23.	**FR3**	+	+
24.	**FR4**	+	-
25.	**FR5**	+	-
26.	**FR6**	+	+
27.	**FR7**	+	-
28.	**FR8**	+	-
29.	**HP1**	+	+
30.	**HP2**	+	+
31.	**HP3**	+	+
32.	**HP4**	+	+
33.	**HP5**	+	+
34.	**HP6**	+	+
35.	**HP7**	+	-
36.	**HP8**	+	-
37.	**HP9**	+	-
38.	**HP10**	+	+
			+= Positive, -= Negative

4.2.2.2 Secondary biochemical test for bacteria isolated from fresh water snail

Once the primary tests confirmed, the bacterial isolates were further subjected to secondary biochemical tests for further identification on the basis of primary test performed (Table 4.4) The result were summarized (Table 4.5 and Plates 28-43).

HSa: HSa was isolated from the visceral tissue/mass of the fresh water snail collected from Water canal near *Satrod* (Hisar). It was Gram positive, facultative anaerobic, fermentive coccus, catalase positive (Plate 26) and oxidase negative with circular shiny colony appearance, entire margin and convex elevation. It showed positive growth for Mueller Hinton agar, Blood agar base no.2, Urea broth base agar, Vogel-Johnson agar, Voges Proskauer medium and Malonate Broth. This bacterium showed negative growth for Luria Bertani agar, Mac-Conkey agar, EMB agar, *Salmonella Shigella* agar, *Pseudomonas* agar F Base, M *Enterococcus* agar, Endo agar, TCBS agar, *Aeromonas* Isolation Base agar, Brilliant Green Agar, Voilet Red Bile Glucose agar and Cetrimide agar. Bacterial isolates were characterized using biochemical test. On the basis of these tests, isolate **HSa** may belong to *Staphylococcus* genus- $\mathbf{I_1}$ (Table 4.5).

HSb: HSb was isolated from the visceral tissue/mass of the fresh water snail collected from Water canal near *Satrod* (Hisar). It was Gram negative, aerobic, fastidious coccus, catalase positive and oxidase positive with circular colony appearance, entire margin and convex elevation. It showed positive growth for Mueller Hinton agar, Blood agar base no.2 and Endo agar. This bacterium showed negative growth for Luria Bertani agar, Mac-Conkey agar, EMB agar, *Salmonella Shigella* agar, *Pseudomonas* agar F Base, M *Enterococcus* agar, Urea broth base agar, Vogel-Johnson agar, Voges Proskauer medium and Malonate Broth, TCBS agar, *Aeromona*s Isolation Base agar, Brilliant Green Agar, Voilet Red Bile Glucose agar and Cetrimide agar. Bacterial isolates were characterized using biochemical test. On the basis of these tests, isolate **HSb** may belong to *Neisseria* genus- $\mathbf{I_1}$ (Table 4.5).

HSx: HSx was isolated from the visceral tissue/mass of the fresh water snail collected from Water canal near *Satrod* (Hisar). It was Gram negative, aerobic, fastidious coccus, catalase positive and oxidase positive (Plate 27) with circular colony appearance, entire margin and convex elevation. It showed positive growth for Mueller Hinton agar, Blood agar base no.2 and Endo agar. This bacterium showed negative growth for Luria Bertani agar, Mac-Conkey agar, EMB agar, *Salmonella Shigella* agar, *Pseudomonas* agar F Base, M *Enterococcus* agar, Urea broth base agar, Vogel-Johnson agar, Voges Proskauer medium and Malonate Broth, TCBS agar, *Aeromonas* Isolation Base agar, Brilliant Green Agar, Voilet Red Bile Glucose agar and Cetrimide agar. Bacterial isolates were characterized using biochemical test. On the basis of these tests, isolate **HSx** may belong to *Neisseria* genus-$\mathbf{I_2}$ (Table 4.5).

4.5 Secondary biochemical tests for bacteria isolated from fresh water snail

Sr. No.	Bacterial isolates	Mac-Conkey agar	EMB agar	SS agar	LB agar	MH agar	BG agar	M-Enterococcus agar	Endo agar	TCBS agar	Blood agar	VRBG agar	Malonate broth	Aeromonas Isolation Base agar	Cetri-mide agar	Pseudo-monas agar	VJ agar	VP medium	Urea Broth
1	HSa	-	-	-	-	+	-	-	-	-	+	-	+	-	-	-	+	+	+
2	HSb	-	-	-	+	+	-	-	+	-	+	-	-	-	-	-	-	-	-
3	HSx	-	-	-	+	+	-	-	+	-	+	-	-	-	-	-	-	-	-
4	HSj	+	+	-	-	+	-	-	+	-	+	+	-	+	-	-	-	-	+
5	HAd	-	-	-	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-
6	HAo	-	-	-	-	+	-	-	-	-	+	-	+	-	-	-	+	+	+
7	HAn	-	-	-	+	+	-	-	-	-	+	-	-	-	-	-	-	-	-
8	HAf	+	+	+	+	+	-	-	+	-	+	+	+	-	-	+	-	+	+
9	HB1	-	+	-	-	+	-	-	+	-	+	-	+	-	-	-	-	-	-
10	HB2	-	+	-	+	+	-	-	+	-	+	+	-	-	+	+	-	-	-
11	HB3	-	-	-	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-
12	HB4	+	+	+	+	+	-	-	+	-	+	+	-	-	-	-	+	-	+
13	HB5	-	-	-	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-
14	HB6	-	-	-	-	+	-	-	-	-	+	-	-	-	-	-	+	-	-
15	HB7	+	+	+	+	+	-	-	+	-	+	+	+	-	-	-	-	+	+
16	HB8	+	+	-	-	+	-	+	+	-	+	-	-	-	-	-	-	-	+
17	HB9	-	-	-	+	+	-	-	-	-	+	-	-	-	-	-	-	-	-
18	HB10	-	+	-	+	+	-	-	+	+	+	+	-	-	+	+	-	-	-
19	HB11	-	-	-	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-
20	HB12	-	+	-	-	+	-	-	-	-	-	-	+	-	-	-	-	-	-
21	FR1	-	-	-	+	+	-	-	-	-	+	-	-	-	-	-	-	-	-
22	FR2	-	-	-	-	+	-	-	-	-	+	-	-	-	-	-	+	-	+
23	FR3	+	+	+	-	+	-	-	+	+	+	+	-	-	+	+	-	-	-
24	FR4	-	-	-	-	+	-	-	-	-	+	-	-	-	-	-	-	+	-
25	FR5	-	-	-	+	+	-	-	-	-	+	-	-	-	-	-	-	-	-
26	FR6	-	-	-	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-
27	FR7	+	+	+	-	+	+	-	+	-	+	+	-	-	-	-	+	-	-
28	FR8	-	-	-	+	+	-	-	-	-	+	-	-	-	-	-	-	-	-
29	HP1	-	+	-	+	+	-	-	+	+	+	+	-	-	+	+	-	-	-
30	HP2	-	+	-	+	+	-	-	+	+	+	+	-	-	+	+	-	-	-
31	HP3	+	+	+	+	+	-	-	+	+	+	+	-	+	-	-	+	-	-
32	HP4	+	+	+	-	+	-	-	+	+	+	+	-	-	+	+	-	-	-
33	HP5	-	+	-	+	+	-	-	+	+	+	+	-	-	+	+	-	-	-
34	HP6	-	+	-	+	+	-	-	+	+	+	+	-	-	+	+	-	-	-
35	HP7	+	+	+	-	+	+	-	+	-	+	+	-	-	-	-	+	-	-
36	HP8	+	-	-	-	+	-	-	-	-	+	-	-	-	-	-	-	+	-
37	HP9	-	-	-	-	+	-	-	-	-	+	-	-	-	-	-	-	+	-
38	HP10	+	+	+	-	+	-	-	+	+	+	+	-	-	+	-	-	-	-

+= Positive, -= Negative

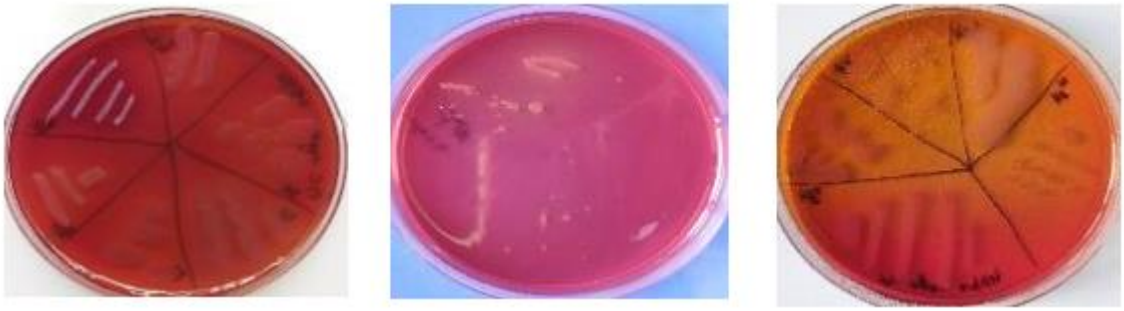


Plate 28: Bacterial isolate(s) grown on differential medium- MacConkey agar

Plate 29: Bacterial isolate(s) grown on differential medium-Eosin Methylene Blue (EMB) agar

Plate 30: Bacterial isolates grown on differential medium-*Salmonella Shigella* (SS)agar

Plate 31: Bacterial isolate(s) grown on differential medium-Luria Bertani (LB) agar

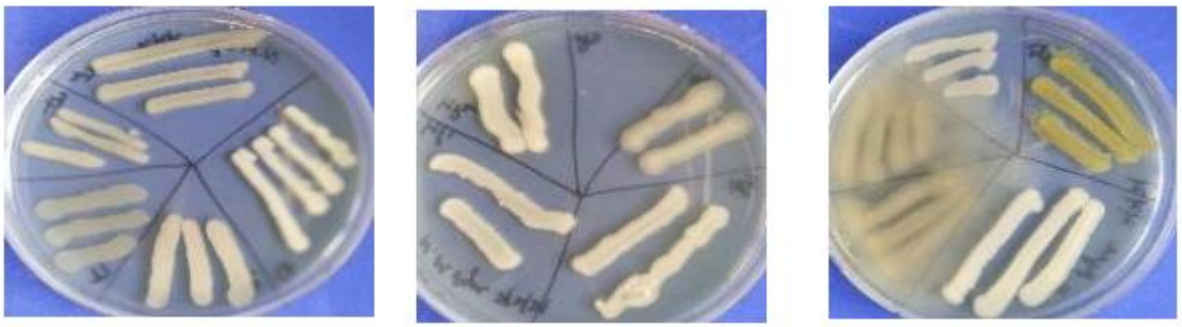


Plate 32: Bacterial isolate(s) grown on differential medium-Mueller Hinton (MH) agar

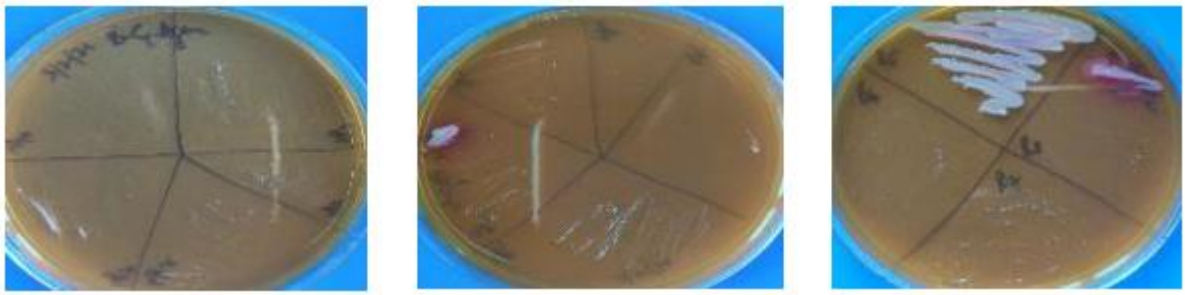


Plate 33: Bacterial isolate(s) grown on differential medium-Brilliant Green (BG) agar

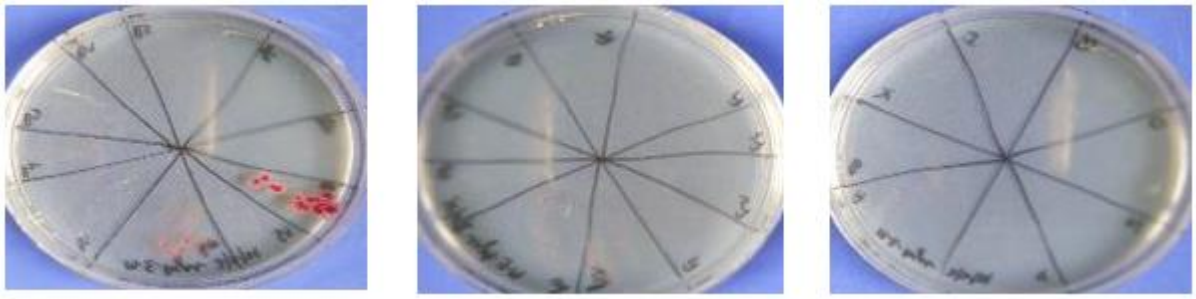


Plate 34: Bacterial isolate(s) grown on differential medium- M-*Enterococcus* agar base

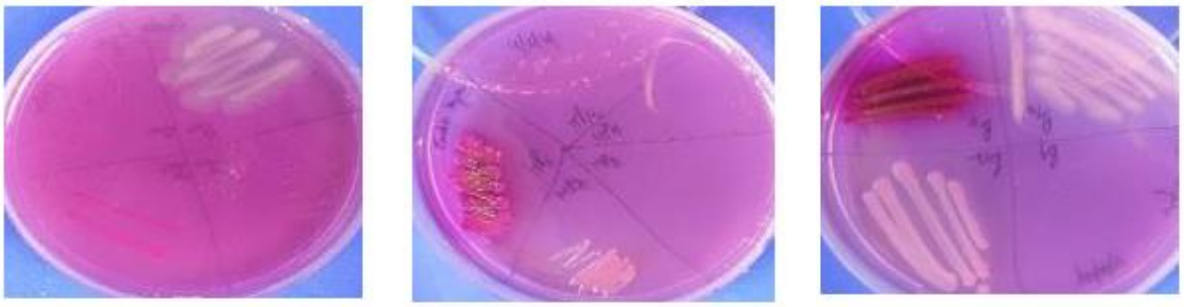


Plate 35: Bacterial isolate(s) grown on differential medium- Endo agar

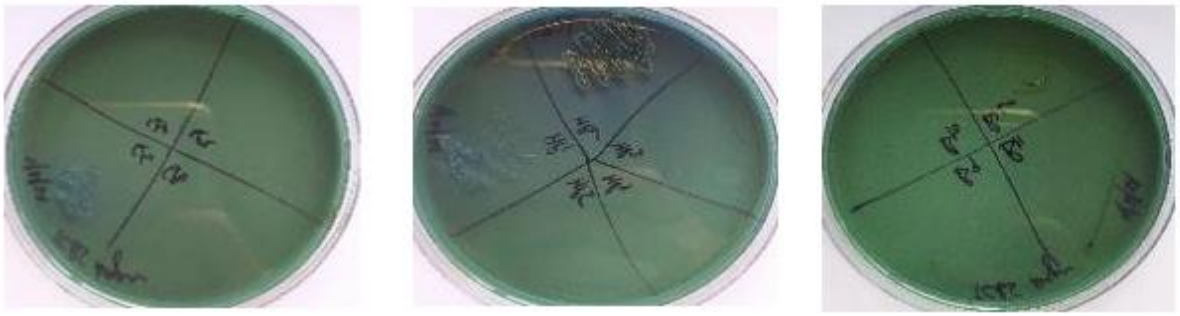


Plate 36: Bacterial isolate(s) grown on differential medium- Thiosulfate Citrate Bile Salt Sucrose (TCBS) agar

Plate 37: Bacterial isolate(s) grown on differential medium-Blood agar base

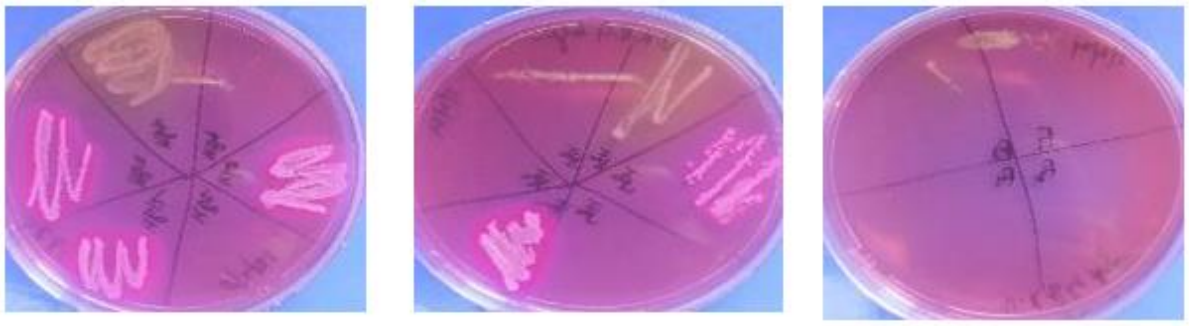


Plate 38: Bacterial isolate(s) grown on differential medium-Violet Red Bile Glucose (VRBG) agar

Plate 39: Bacterial isolate(s) grown on differential medium- Malonate agar

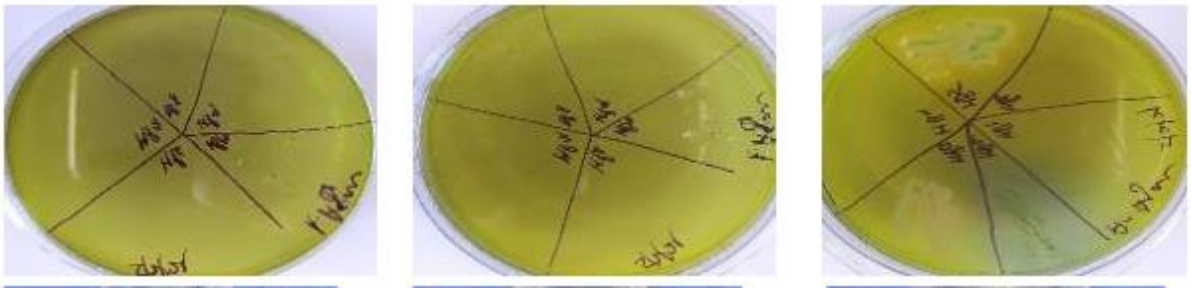


Plate 40: Bacterial isolate(s) grown on differential medium- *Aeromonas* isolation medium base

Plate 41: Bacterial isolate(s) grown on differential medium- Cetrimide agar

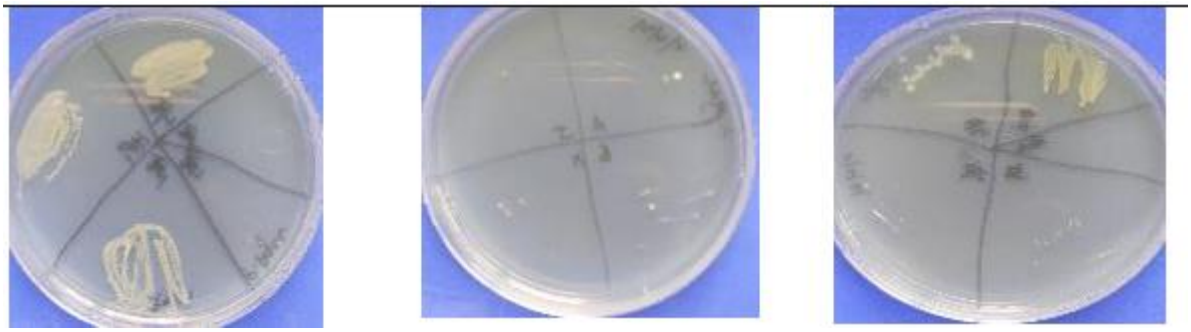
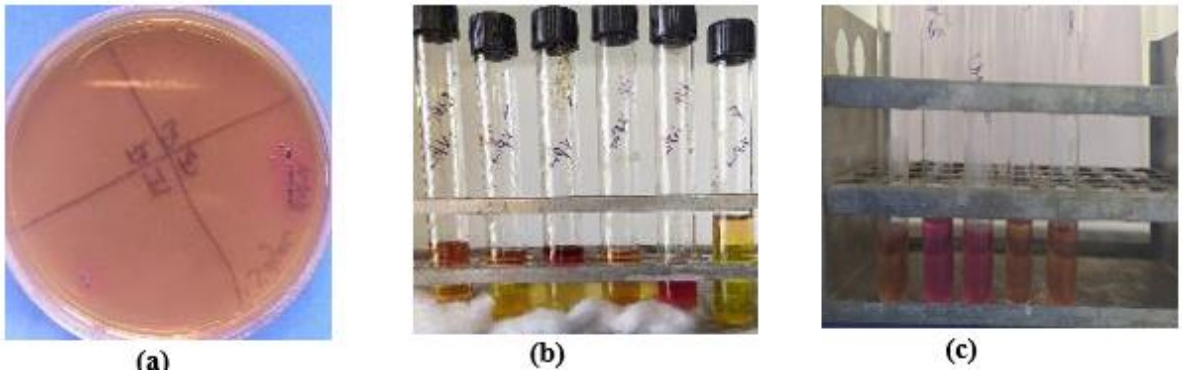


Plate 42: Bacterial isolate(s) grown on differential medium- *Pseudomonas* agar base



(a) (b) (c)

Plate 43: Bacterial isolate(s) grown on differential medium-(a)Vogel Johnson agar base, (b)Voges Proskauer medium & (c)Urea broth base agar

Bacterial isolate(s) grown on various differential medium

HSj: HSj was isolated from the visceral tissue/mass of the fresh water snail collected from Water canal near *Satrod* (Hisar). It was Gram negative, facultative anaerobic, bacillus and oxidase negative with circular shiny colony appearance, entire margin and convex shiny elevation. It showed positive growth for Mac-Conkey agar, EMB agar, Voilet Red Bile Glucose agar, Urea broth base agar, Mueller Hinton agar, *Aeromonas* Isolation Base agar, Blood agar base no.2 and Endo agar. This bacterium showed negative growth for Luria Bertani agar, *Salmonella Shigella* agar, *Pseudomonas* agar F Base, M *Enterococcus* agar, Vogel-Johnson agar, Voges Proskauer medium, Malonate Broth, TCBS agar, Brilliant Green Agar and Cetrimide agar. Bacterial isolates were characterized using biochemical test. On the basis of these tests, isolate **HSj** may belong to *Yersinia* genus (Table 4.5).

HAd: HAd was isolated from the visceral tissue/mass of the fresh water snail collected from Water canal near *Azad nagar* (Hisar). It was Gram positive, aerobic, coccus, catalase positive (Plate 26) and oxidase positive with circular colony appearance, entire margin and convex elevation. It showed positive growth for Mueller Hinton agar, Blood agar base no.2. This bacterium showed negative growth for Luria Bertani agar, Mac-Conkey agar, EMB agar, *Salmonella Shigella* agar, *Pseudomonas* agar F Base, M *Enterococcus* agar, Endo agar, TCBS agar, *Aeromonas* Isolation Base agar, Brilliant Green Agar, Urea broth base agar, Vogel-Johnson agar, Voges Proskauer medium, Malonate Broth, Voilet Red Bile Glucose agar and Cetrimide agar. Bacterial isolates were characterized using biochemical test. On the basis of these tests, isolate **HAd** may belong to *Micrococcus* genus- $\mathbf{I_1}$ (Table 4.5).

HAo: HAo was isolated from the visceral tissue/mass of the fresh water snail collected from Water canal near *Azad nagar* (Hisar). It was Gram positive, facultative anaerobic, fermentive coccus, catalase positive (Plate 26) and oxidase negative with circular shiny colony appearance, entire margin and convex elevation. It showed positive growth for Mueller Hinton agar, Blood agar base no.2, Urea broth base agar, Vogel-Johnson agar, Voges Proskauer medium and Malonate Broth. This bacterium showed negative growth for Luria Bertani agar, Mac-Conkey agar, EMB agar, *Salmonella Shigella* agar, *Pseudomonas* agar F Base, M *Enterococcus* agar, Endo agar, TCBS agar, *Aeromonas* Isolation Base agar, Brilliant Green Agar, Voilet Red Bile Glucose agar and Cetrimide agar. Bacterial isolates were characterized using biochemical test. On the basis of these tests, isolate **HAo** may belong to *Staphylococcus* genus- $\mathbf{I_2}$ (Table 4.5).

HAn: HAn was isolated from the visceral tissue/mass of the fresh water snail collected from Water canal near *Azad nagar* (Hisar). It was Gram positive, facultative anaerobic, spore forming bacillus, catalase positive (Plate 26) and oxidase negative with circular colony appearance, entire margin and convex

elevation. It showed positive growth for Luria Bertani agar, Mueller Hinton agar and Blood agar base no.2. This bacterium showed negative growth for Mac-Conkey agar, EMB agar, *Salmonella Shigella* agar, *Pseudomonas* agar F Base, M *Enterococcus* agar, Endo agar, TCBS agar, *Aeromonas* Isolation agar base, Brilliant Green Agar, Voilet Red Bile Glucose agar, Urea broth base agar, Vogel-Johnson agar, Voges Proskauer medium, Malonate Broth and Cetrimide agar. Bacterial isolates were characterized using biochemical test. On the basis of these tests, isolate **HAn** may belong to C*lostridium* genus- **I_1** (Table 4.5).

HAf: The bacterium HAf was isolated from the visceral tissue/mass of the fresh water snail collected from Water canal near *Azad nagar* (Hisar). It was Gram negative, facultative anaerobic, non fermentive rods, catalase positive and oxidase negative with circular colony appearance, entire margin and convex elevation. This was positive for Mac-Conkey agar, EMB agar, Urea Broth base agar, Voilet Red Bile Glucose agar, *Salmonella Shigella* agar, Luria Bertani agar, Endo agar, Voges Proskauer medium, *Pseudomonas* agar F Base, Mueller Hinton agar, Blood agar base no.2 and Malonate Broth. This bacterium showed negative growth for Vogel-Johnson agar, M *Enterococcus* agar, Brilliant Green Agar, TCBS agar, Cetrimide agar and *Aeromonas* Isolation agar base. Bacterial isolates were characterized using biochemical test. On the basis of these tests, isolate **HAf** may belong to *Enterobacter* genus - **I_1** (Table 4.5).

HB_1: The bacterium HB_1 was isolated also from the visceral tissue/mass of the fresh water snail collected from water canal near *Balsamand* road, (Hisar). It was Gram negative, facultative anaerobic, non fermentive coccus, catalase positive and oxidase positive with irregular colony appearance, lobate margin and flat elevation. This showed positive growth for EMB agar, Mueller Hinton agar, Endo agar, Malonate Broth and Blood agar base no.2. This bacterium showed negative growth for Luria Bertani agar, Voges Proskauer medium, *Pseudomonas* agar F Base, Brilliant Green Agar, Voilet Red Bile Glucose agar, Vogel-Johnson agar, M *Enterococcus* agar, TCBS agar, Cetrimide agar, *Salmonella Shigella* agar, *Aeromonas* Isolation agar base, Mac-Conkey agar and Urea Broth base agar. Bacterial isolates were characterized using biochemical test. On the basis of these tests, isolate **HB_1** may be *Campylobacter* genus- **I_1** (Table 4.5).

HB_2: The bacterium HB_2 was isolated from the visceral tissue/mass of the fresh water snail collected from water canal near *Balsamand* road, (Hisar). It was Gram negative, aerobic coccus and oxidase positive bacteria with circular colony appearance, entire margin and raised elevation. This showed positive growth for EMB agar, Voilet Red Bile Glucose agar, Mueller Hinton agar, Endo agar, TCBS agar, Luria Bertani agar, Blood agar base no.2 and *Pseudomonas* agar F Base. This bacterium showed negative growth for Malonate Broth, Voges Proskauer medium, Vogel-Johnson agar, M *Enterococcus* agar, Brilliant Green

Agar, Cetrimide agar, *Salmonella Shigella* agar, *Aeromonas* Isolation agar base, Mac-Conkey agar and Urea Broth base agar. Bacterial isolates were characterized using biochemical test. On the basis of these tests, isolate **HB_2** may be *Pseudomonas* genus$_1$- **I_1** (Table 4.5).

HB_3: HB_3 was isolated from the visceral tissue/mass of the fresh water snail collected from water canal near *Balsamand* road, (Hisar). It was Gram positive, aerobic, coccus, catalase positive and oxidase positive (Plate 27) with circular colony appearance, entire margin and convex elevation. It showed positive growth for Mueller Hinton agar, Blood agar base no.2. This bacterium showed negative growth for Luria Bertani agar, Mac-Conkey agar, EMB agar, *Salmonella Shigella* agar, *Pseudomonas* agar F Base, M *Enterococcus* agar, Endo agar, TCBS agar, *Aeromonas* Isolation Base agar, Brilliant Green Agar, Urea broth base agar, Vogel-Johnson agar, Voges Proskauer medium, Malonate Broth, Voilet Red Bile Glucose agar and Cetrimide agar. Bacterial isolates were characterized using biochemical test. On the basis of these tests, isolate **HB_3** may belong to *Micrococcus* genus- **I_2** (Table 4.5).

HB_4: HB_4 was isolated from the visceral tissue/mass of the fresh water snail collected from water canal near *Balsamand* road, (Hisar). It was Gram negative, facultative anaerobic, bacillus, catalase positive (Plate 26) and oxidase negative with circular colony appearance, entire margin and convex elevation. It showed positive growth for Mueller Hinton agar, Luria Bertani agar, Mac-Conkey agar, EMB agar, *Salmonella Shigella* agar, Urea broth base agar, Vogel-Johnson agar, Voilet Red Bile Glucose agar, Endo agar and Blood agar base no.2. This bacterium showed negative growth for *Pseudomonas* agar F Base, M *Enterococcus* agar, TCBS agar, *Aeromonas* Isolation Base agar, Brilliant Green Agar, Voges Proskauer medium, Malonate Broth and Cetrimide agar. Bacterial isolates were characterized using biochemical test. On the basis of these tests, isolate **HB_4** may belong to *Proteus* genus (Table 4.5).

HB_5: HB_5 was isolated from the visceral tissue/mass of the fresh water snail collected from water canal near *Balsamand* road, (Hisar). It was Gram positive, aerobic, coccus, catalase positive (Plate 26) and oxidase positive with circular colony appearance, entire margin and convex elevation. It showed positive growth for Mueller Hinton agar, Blood agar base no.2. This bacterium showed negative growth for Luria Bertani agar, Mac-Conkey agar, EMB agar, *Salmonella Shigella* agar, *Pseudomonas* agar F Base, M *Enterococcus* agar, Endo agar, TCBS agar, *Aeromonas* Isolation Base agar, Brilliant Green Agar, Urea broth base agar, Vogel-Johnson agar, Voges Proskauer medium, Malonate Broth, Voilet Red Bile Glucose agar and Cetrimide agar. Bacterial isolates were characterized using biochemical test. On the basis of these tests, isolate **HB_5** may belong to *Micrococcus* genus- **I_3** (Table 4.5).

HB_6: HB_6 was isolated from the visceral tissue/mass of the fresh water snail collected from water canal near *Balsamand* road, (Hisar). It was Gram positive, facultative anaerobic, cocci shape long chain, catalase negative and oxidase negative bacteria with circular colony appearance, entire margin and flat elevation. It showed positive growth for Mueller Hinton agar, Vogel-Johnson agar and Blood agar base no.2. This bacterium showed negative growth for Luria Bertani agar, Mac-Conkey agar, EMB agar, *Salmonella Shigella* agar, *Pseudomonas* agar F Base, M *Enterococcus* agar, Endo agar, TCBS agar, *Aeromonas* Isolation Base agar, Brilliant Green Agar, Urea broth base agar, Voges Proskauer medium, Malonate Broth, Voilet Red Bile Glucose agar and Cetrimide agar. Bacterial isolates were characterized using biochemical test. On the basis of these tests, isolate **HB_6** may belong to *Streptococcus* genus (Table 4.5).

HB_7: The bacterium HB_7 was isolated from the visceral tissue/mass of the fresh water snail collected from water canal near *Balsamand* road, (Hisar). It was Gram negative, facultative anaerobic, non fermentive rods, catalase positive and oxidase negative with circular colony appearance, entire margin and convex elevation. This showed positive growth for Mac-Conkey agar, EMB agar, Urea Broth base agar, Voilet Red Bile Glucose agar, *Salmonella Shigella* agar, Luria Bertani agar, Endo agar, Voges Proskauer medium, *Pseudomonas* agar F Base, Mueller Hinton agar, Blood agar base no.2 and Malonate Broth. This bacterium showed negative growth for Vogel-Johnson agar, M *Enterococcus* agar, Brilliant Green Agar, TCBS agar, Cetrimide agar and *Aeromonas* Isolation agar base. Bacterial isolates were characterized using biochemical test. On the basis of these tests, isolate **HB_7** may belong to *Enterobacter* genus – **I_2** (Table 4.5).

HB_8: The bacterium HB_8 was isolated from the visceral tissue/mass of the fresh water snail collected from water canal near *Balsamand* road, (Hisar). It was Gram positive, facultative anaerobic, cocci shape, catalase negative and oxidase negative with irregular colony appearance, entire margin and flat elevation. This showed positive growth for M *Enterococcus* agar, Mac-Conkey agar, EMB agar, Urea Broth base agar, Endo agar, Mueller Hinton agar and Blood agar base no.2. This bacterium showed negative growth for Voilet Red Bile Glucose agar, *Salmonella Shigella* agar, Luria Bertani agar, Vogel-Johnson agar, Brilliant Green Agar, TCBS agar, Voges Proskauer medium, *Pseudomonas* agar F Base, Malonate Broth Cetrimide agar and *Aeromonas* Isolation agar base. Bacterial isolates were characterized using biochemical test. On the basis of these tests, isolate **HB_8** may belong to *Enterococcus* genus (Table 4.5).

HB_9: HB_9 was isolated from the visceral tissue/mass of the fresh water snail collected from canal near *Balsamand* road, Hisar. It was Gram positive, facultative anaerobic, spore forming bacillus, catalase positive and oxidase negative (Plate 27) with circular colony appearance, entire margin and convex elevation. It showed positive growth for Luria Bertani agar, Mueller Hinton agar and Blood agar base no.2.

This bacterium showed negative growth for Mac-Conkey agar, EMB agar, Salmonella Shigella agar, Pseudomonas agar F Base, M Enterococcus agar, Endo agar, TCBS agar, Aeromonas Isolation agar base, Brilliant Green Agar, Voilet Red Bile Glucose agar, Urea broth base agar, Vogel-Johnson agar, Voges Proskauer medium, Malonate Broth and Cetrimide agar. Bacterial isolates were characterized using biochemical test. On the basis of these tests, isolate **HB_9** may belong to C*lostridium* genus- **I_2** (Table 4.5).

HB_{10}: The bacterium HB_{10} was isolated also from the visceral tissue/mass of the fresh water snail collected from canal near *Balsamand* road, Hisar. It was Gram negative, aerobic (at times facultative anaerobic), coccus and oxidase positive bacteria with circular colony appearance, entire margin and raised elevation. This showed positive growth for EMB agar, Voilet Red Bile Glucose agar, Mueller Hinton agar, Endo agar, TCBS agar, Luria Bertani agar, Blood agar base no.2 and *Pseudomonas* agar F Base. This bacterium showed negative growth for Malonate Broth, Voges Proskauer medium, Vogel-Johnson agar, M *Enterococcus* agar, Brilliant Green Agar, Cetrimide agar, *Salmonella Shigella* agar, *Aeromonas* Isolation agar base, Mac-Conkey agar and Urea Broth base agar. Bacterial isolates were characterized using biochemical test. On the basis of these tests, isolate **HB_{10}** may be *Pseudomonas* $genus_1$- **I_2** (Table 4.5).

HB_{11}: HB_{11} was isolated from the visceral tissue/mass of the fresh water snail collected from water canal near *Balsamand* road, (Hisar). It was Gram positive, aerobic, coccus, catalase positive (Plate 26) and oxidase positive with circular colony appearance, entire margin and convex elevation. It showed positive growth for Mueller Hinton agar, Blood agar base no.2. This bacterium showed negative growth for Luria Bertani agar, Mac-Conkey agar, EMB agar, *Salmonella Shigella* agar, *Pseudomonas* agar F Base, M *Enterococcus* agar, Endo agar, TCBS agar, *Aeromonas* Isolation Base agar, Brilliant Green Agar, Urea broth base agar, Vogel-Johnson agar, Voges Proskauer medium, Malonate Broth, Voilet Red Bile Glucose agar and Cetrimide agar. Bacterial isolates were characterized using biochemical test. On the basis of these tests, isolate **HB_{11}** may belong to *Micrococcus* genus- **I_4** (Table 4.5).

HB_{12}: The bacterium HB_{12} was isolated also from the visceral tissue/mass of the fresh water snail collected from water canal near *Balsamand* road, (Hisar). It was Gram negative, facultative anaerobic, non fermentive coccus, catalase positive and oxidase positive with Irregular colony appearance, lobate margin and flat elevation. This showed positive growth for EMB agar, Mueller Hinton agar, Endo agar, Malonate Broth and Blood agar base no.2. This bacterium showed negative growth for Luria Bertani agar, Voges Proskauer medium, *Pseudomonas* agar F Base, Brilliant Green Agar, Voilet Red Bile Glucose agar, Vogel-Johnson agar, M *Enterococcus* agar, TCBS agar, Cetrimide agar, *Salmonella Shigella* agar, *Aeromonas* Isolation agar base, Mac-Conkey agar and Urea Broth base agar. Bacterial isolates were characterized

using biochemical test. On the basis of these tests, isolate **HB_{12}** may be *Campylobacter* genus- **I_2** (Table 4.5).

FR_1: FR_1 was isolated from the visceral tissue/mass of the fresh water snail collected from rice fields of district *Fatehabad*. It was Gram positive, facultative anaerobic, spore forming bacillus, catalase positive and oxidase negative (Plate 27) with circular colony appearance, entire margin and convex elevation. It showed positive growth for Luria Bertani agar, Mueller Hinton agar and Blood agar base no.2. This bacterium showed negative growth for Mac-Conkey agar, EMB agar, *Salmonella Shigella* agar, *Pseudomonas* agar F Base, M *Enterococcus* agar, Endo agar, TCBS agar, *Aeromonas* Isolation agar base, Brilliant Green Agar, Voilet Red Bile Glucose agar, Urea broth base agar, Vogel-Johnson agar, Voges Proskauer medium, Malonate Broth and Cetrimide agar. Bacterial isolates were characterized using biochemical test. On the basis of these tests, isolate **FR_1** may belong to C*lostridium* genus- **I_3** (Table 4.5).

FR_2: FR_2 was isolated from the visceral tissue/mass of the fresh water snail collected from collected from rice fields of district *Fatehabad*. It was Gram positive, facultative anaerobic, fermentive coccus, catalase positive (Plate 26) and oxidase negative with circular shiny colony appearance, entire margin and convex elevation. It showed positive growth for Mueller Hinton agar, Blood agar base no.2, Urea broth base agar, Vogel-Johnson agar, Voges Proskauer medium and Malonate Broth. This bacterium showed negative growth for Luria Bertani agar, Mac-Conkey agar, EMB agar, *Salmonella Shigella* agar, *Pseudomonas* agar F Base, M *Enterococcus* agar, Endo agar, TCBS agar, *Aeromonas* Isolation Base agar, Brilliant Green Agar, Voilet Red Bile Glucose agar and Cetrimide agar. Bacterial isolates were characterized using biochemical test. On the basis of these tests, isolate **FR_2** may belong to *Staphylococcus* genus- **I_3** (Table 4.5).

FR_3: FR_3 was isolated from the visceral tissue/mass of the fresh water snail collected from collected from rice fields of district *Fatehabad*. It was Gram positive, strict aerobic, fermentive rods, catalase positive (Plate 26) and oxidase positive with circular colony appearance, entire margin and convex shiny elevation. It showed positive growth for Mac-Conkey agar, Cetrimide agar, Voilet Red Bile Glucose agar, Mueller Hinton agar, Endo agar, TCBS agar, *Pseudomonas* agar F Base, *Salmonella Shigella* agar and Blood agar base no.2. This bacterium showed negative growth for Luria Bertani agar, EMB agar, Urea broth base agar, M *Enterococcus* agar, Voges Proskauer medium, Malonate Broth, *Aeromonas* Isolation Base agar, Brilliant Green Agar and Vogel-Johnson agar. Bacterial isolates were characterized using biochemical test. On the basis of these tests, isolate **FR_3** may belong to *Pseudomonas* $genus_2$- **I_1** (Table 4.5).

FR_4: FR_4 was isolated from the visceral tissue/mass of the fresh water snail collected from collected from rice fields of district *Fatehabad*. It was Gram positive, strict aerobic, fermentive rods, catalase positive (Plate 26) and oxidase negative with irregular colony appearance, lobate margin and flat elevation. It showed positive growth for Voges Proskauer medium, Blood agar base no.2 and Mueller Hinton agar. This bacterium showed negative growth for Mac-Conkey agar, Cetrimide agar, Voilet Red Bile Glucose agar, Luria Bertani agar, EMB agar, Urea broth base agar, Endo agar, TCBS agar, *Pseudomonas* agar F Base, *Salmonella Shigella* agar, M *Enterococcus* agar, Malonate Broth, *Aeromonas* Isolation Base agar, Brilliant Green Agar and Vogel-Johnson agar. Bacterial isolates were characterized using biochemical test. On the basis of these tests, isolate **FR_4** may belong to *Bacillus* genus-**I_1** (Table 4.5).

FR_5: FR_5 was isolated from the visceral tissue/mass of the fresh water snail collected from rice fields of district *Fatehabad*. It was Gram positive, facultative anaerobic, spore forming bacillus, catalase positive and oxidase negative with circular colony appearance, entire margin and convex elevation. It showed positive growth for Luria Bertani agar, Mueller Hinton agar and Blood agar base no.2. This bacterium showed negative growth for Mac-Conkey agar, EMB agar, *Salmonella Shigella* agar, *Pseudomonas* agar F Base, M *Enterococcus* agar, Endo agar, TCBS agar, *Aeromonas* Isolation agar base, Brilliant Green Agar, Voilet Red Bile Glucose agar, Urea broth base agar, Vogel-Johnson agar, Voges Proskauer medium, Malonate Broth and Cetrimide agar. Bacterial isolates were characterized using biochemical test. On the basis of these tests, isolate **FR_5** may belong to C*lostridium* genus- **I_4** (Table 4.5).

FR_6: FR_6 was isolated from the visceral tissue/mass of the fresh water snail collected from rice fields of district *Fatehabad*. It was Gram positive, aerobic, coccus, catalase positive and oxidase positive (Plate 27) with circular colony appearance, entire margin and convex elevation. It showed positive growth for Mueller Hinton agar, Blood agar base no.2. This bacterium showed negative growth for Luria Bertani agar, Mac-Conkey agar, EMB agar, *Salmonella Shigella* agar, *Pseudomonas* agar F Base, M *Enterococcus* agar, Endo agar, TCBS agar, *Aeromonas* Isolation Base agar, Brilliant Green Agar, Urea broth base agar, Vogel-Johnson agar, Voges Proskauer medium, Malonate Broth, Voilet Red Bile Glucose agar and Cetrimide agar. Bacterial isolates were characterized using biochemical test. On the basis of these tests, isolate **FR_6** may belong to *Micrococcus* genus- **I_5** (Table 4.5).

FR_7: FR_7 was isolated from the visceral tissue/mass of the fresh water snail collected from rice fields of district *Fatehabad*. It was Gram negative, strict aerobic, fermentive rods, catalase positive (Plate 26) and oxidase negative with circular shiny colony appearance, entire margin and convex elevation. It showed positive growth for Endo agar, Brilliant Green Agar, Mac-Conkey agar, Voilet Red Bile Glucose agar,

Vogel-Johnson agar, *Salmonella Shigella* agar, EMB agar, Mueller Hinton agar, Blood agar base no.2. This bacterium showed negative growth for Luria Bertani agar, *Pseudomonas* agar F Base, M *Enterococcus* agar, TCBS agar, *Aeromonas* Isolation Base agar, Urea broth base agar, Voges Proskauer medium, Malonate Broth and Cetrimide agar. Bacterial isolates were characterized using biochemical test. On the basis of these tests, isolate **FR_7** may belong to *Salmonella* genus- **I_1** (Table 4.5).

FR_8: FR_8 was isolated from the visceral tissue/mass of the fresh water snail collected from rice fields of district *Fatehabad*. It was Gram positive, facultative anaerobic, spore forming bacillus, catalase positive and oxidase negative with circular colony appearance, entire margin and convex elevation. It showed positive growth for Luria Bertani agar, Mueller Hinton agar and Blood agar base no.2. This bacterium showed negative growth for Mac-Conkey agar, EMB agar, *Salmonella Shigella* agar, *Pseudomonas* agar F Base, M *Enterococcus* agar, Endo agar, TCBS agar, *Aeromonas* Isolation agar base, Brilliant Green Agar, Voilet Red Bile Glucose agar, Urea broth base agar, Vogel-Johnson agar, Voges Proskauer medium, Malonate Broth and Cetrimide agar. Bacterial isolates were characterized using biochemical test. On the basis of these tests, isolate **FR_8** may belong to C*lostridium* genus- **I_5** (Table 4.5).

HP_1: The bacterium HP_1 was isolated also from the visceral tissue/mass of the fresh water snail collected from Lotus Pond, CCS HAU Hisar. It was Gram negative, aerobic, coccus and oxidase positive bacteria with circular colony appearance, entire margin and raised elevation. This showed positive growth for EMB agar, Voilet Red Bile Glucose agar, Mueller Hinton agar, Endo agar, TCBS agar, Luria Bertani agar, Blood agar base no.2 and *Pseudomonas* agar F Base. This bacterium showed negative growth for Malonate Broth, Voges Proskauer medium, Vogel-Johnson agar, M *Enterococcus* agar, Brilliant Green Agar, Cetrimide agar, *Salmonella Shigella* agar, *Aeromonas* Isolation agar base, Mac-Conkey agar and Urea Broth base agar. Bacterial isolates were characterized using biochemical test. On the basis of these tests, isolate **HP_1** may be *Pseudomonas* $genus_1$- **I_3** (Table 4.5).

HP_2: The bacterium HP_2 was isolated also from the visceral tissue/mass of the fresh water snail collected from Lotus Pond, CCS HAU Hisar. It was Gram negative, aerobic, coccus and oxidase positive bacteria with circular colony appearance, entire margin and raised elevation. This was positive for EMB agar, Voilet Red Bile Glucose agar, Mueller Hinton agar, Endo agar, TCBS agar, Luria Bertani agar, Blood agar base no.2 and *Pseudomonas* agar F Base. This bacterium showed negative growth for Malonate Broth, Voges Proskauer medium, Vogel-Johnson agar, M *Enterococcus* agar, Brilliant Green Agar, Cetrimide agar, *Salmonella Shigella* agar, *Aeromonas* Isolation agar base, Mac-Conkey agar and Urea Broth base

agar. Bacterial isolates were characterized using biochemical test. On the basis of these tests, isolate **HP_2** may be *Pseudomonas* genus$_1$- **I_4** (Table 4.5).

HP_3: The bacterium HP_3 was isolated also from the visceral tissue/mass of the fresh water snail collected from Lotus Pond, CCS HAU Hisar. It was Gram negative, facultative anaerobic, rod shape, catalase positive and oxidase positive bacteria with irregular colony appearance, ungulate margin and umbonate elevation. This showed positive growth for Blood agar base no.2, Vogel-Johnson agar, *Aeromonas* Isolation agar base, Mac-Conkey agar, *Salmonella Shigella* agar, Endo agar, TCBS agar, Luria Bertani agar, Mueller Hinton agar Urea Broth base agar, EMB agar, Voilet Red Bile Glucose agar, and *Pseudomonas* agar F Base. This bacterium showed negative growth for Malonate Broth, Voges Proskauer medium, M *Enterococcus* agar, Brilliant Green Agar and Cetrimide agar. Bacterial isolates were characterized using biochemical test. On the basis of these tests, isolate **HP_3** may be *Aeromonas* genus (Table 4.5).

HP_4: HP_4 was isolated from the visceral tissue/mass of the fresh water snail collected from Lotus Pond, CCS HAU Hisar It was Gram positive, strict aerobic, fermentive rods, catalase positive (Plate 26) and oxidase positive with circular colony appearance, entire margin and convex shiny elevation. It showed positive growth for Mac-Conkey agar, Cetrimide agar, Voilet Red Bile Glucose agar, Mueller Hinton agar, Endo agar, TCBS agar, *Pseudomonas* agar F Base, *Salmonella Shigella* agar and Blood agar base no.2. This bacterium showed negative growth for Luria Bertani agar, EMB agar, Urea broth base agar, M *Enterococcus* agar, Voges Proskauer medium, Malonate Broth, Aeromonas Isolation Base agar, Brilliant Green Agar and Vogel-Johnson agar Bacterial isolates were characterized using biochemical test. On the basis of these tests, isolate **HP_4** may belong to *Pseudomonas* genus$_2$- **I_2** (Table 4.5).

HP_5: The bacterium HP_5 was isolated also from the visceral tissue/mass of the fresh water snail collected from Lotus Pond, CCS HAU Hisar. It was Gram negative, aerobic, coccus and oxidase positive bacteria with circular colony appearance, entire margin and raised elevation. This was positive for EMB agar, Voilet Red Bile Glucose agar, Mueller Hinton agar, Endo agar, TCBS agar, Luria Bertani agar, Blood agar base no.2 and *Pseudomonas* agar F Base. This bacterium showed negative growth for Malonate Broth, Voges Proskauer medium, Vogel-Johnson agar, M *Enterococcus* agar, Brilliant Green Agar, Cetrimide agar, *Salmonella Shigella* agar, *Aeromonas* Isolation agar base, Mac-Conkey agar and Urea Broth base agar. Bacterial isolates were characterized using biochemical test. On the basis of these tests, isolate **HP_5** may be *Pseudomonas* genus$_1$- **I_5** (Table 4.5).

HP_6: The bacterium HP_6 was isolated also from the visceral tissue/mass of the fresh water snail collected from Lotus Pond, CCS HAU Hisar. It was Gram negative, aerobic coccus and oxidase positive bacteria with circular colony appearance, entire margin and raised elevation. This showed positive growth for EMB agar, Voilet Red Bile Glucose agar, Mueller Hinton agar, Endo agar, TCBS agar, Luria Bertani agar, Blood agar base no.2 and *Pseudomonas* agar F Base. This bacterium showed negative growth for Malonate Broth, Voges Proskauer medium, Vogel-Johnson agar, M *Enterococcus* agar, Brilliant Green Agar, Cetrimide agar, *Salmonella Shigella* agar, *Aeromonas* Isolation agar base, Mac-Conkey agar and Urea Broth base agar. Bacterial isolates were characterized using biochemical test. On the basis of these tests, isolate **HP_6** may be *Pseudomonas* $genus_1$- **I_6** (Table 4.5).

HP_7: HP_7 was isolated from the visceral tissue/mass of the fresh water snail collected from Lotus Pond, CCS HAU Hisar. It was Gram negative, strict aerobic, fermentive rods, catalase positive (Plate 26) and oxidase negative with circular shiny colony appearance, entire margin and convex elevation. It showed positive growth for Endo agar, Brilliant Green Agar, Mac-Conkey agar, Voilet Red Bile Glucose agar, Vogel-Johnson agar, *Salmonella Shigella* agar, EMB agar, Mueller Hinton agar, Blood agar base no.2. This bacterium showed negative growth for Luria Bertani agar, *Pseudomonas* agar F Base, M *Enterococcus* agar, TCBS agar, *Aeromonas* Isolation Base agar, Urea broth base agar, Voges Proskauer medium, Malonate Broth and Cetrimide agar. Bacterial isolates were characterized using biochemical test. On the basis of these tests, isolate **HP_7** may belong to *Salmonella* genus- **I_2** (Table 4.5).

HP_8: HP_8 was isolated from the visceral tissue/mass of the fresh water snail collected from Lotus Pond, CCS HAU Hisar. It was Gram positive, anaerobic, spore forming bacillus, catalase positive and oxidase negative (Plate 27) with irregular colony appearance, filamentous margin and flat elevation. It showed positive growth for Mac-Conkey agar, Blood agar base no.2, Voges Proskauer medium and Mueller Hinton agar. This bacterium showed negative growth for Luria Bertani agar, EMB agar, *Salmonella Shigella* agar, *Pseudomonas* agar F Base, M *Enterococcus* agar, Endo agar, TCBS agar, *Aeromonas* Isolation agar base, Brilliant Green Agar, Voilet Red Bile Glucose agar, Urea broth base agar, Vogel-Johnson agar, Malonate Broth and Cetrimide agar. Bacterial isolates were characterized using biochemical test. On the basis of these tests, isolate **HP_8** may belong to *Bacillus* genus-**I_2** (Table 4.5).

HP_9: HP_9 was isolated from the visceral tissue/mass of the fresh water snail collected from Lotus Pond, CCS HAU Hisar. It was Gram positive, anaerobic, spore forming bacillus with long chain, catalase positive (Plate 26) and oxidase negative with irregular colony appearance, lobate margin and flat elevation. It showed positive growth for Blood agar base no.2, Voges Proskauer medium and Mueller Hinton agar. This

bacterium showed negative growth for Mac-Conkey agar, Luria Bertani agar, EMB agar, *Salmonella Shigella* agar, *Pseudomonas* agar F Base, M *Enterococcus* agar, Endo agar, TCBS agar, *Aeromonas* Isolation agar base, Brilliant Green Agar, Voilet Red Bile Glucose agar, Urea broth base agar, Vogel-Johnson agar, Malonate Broth and Cetrimide agar. Bacterial isolates were characterized using biochemical test. On the basis of these tests, isolate **HP_9** may belong to *Bacillus* genus-**I_3** (Table 4.5).

HP_{10}: HP_{10} was isolated from the visceral tissue/mass of the fresh water snail collected from Lotus Pond, CCS HAU Hisar. It was Gram positive, strict aerobic, fermentive rods, catalase positive and oxidase positive with circular colony appearance, entire margin and convex shiny elevation. It showed positive growth for Mac-Conkey agar, Cetrimide agar, Voilet Red Bile Glucose agar, Mueller Hinton agar, Endo agar, TCBS agar, *Pseudomonas* agar F Base, *Salmonella Shigella* agar and Blood agar base no.2. This bacterium showed negative growth for Luria Bertani agar, EMB agar, Urea broth base agar, M *Enterococcus* agar, Voges Proskauer medium, Malonate Broth, *Aeromonas* Isolation Base agar, Brilliant Green Agar and Vogel-Johnson agar. Bacterial isolates were characterized using biochemical test. On the basis of these tests, isolate **HP_{10}** may belong to *Pseudomonas* $genus_2$- **I_3** (Table 4.5).

4.3 Evaluation of antimicrobial activity of various essential oils against bacterial isolates

These studies were carried out in Rodentology laboratory, Department of Zoology & Aquaculture, CCS Haryana Agricultural University, Hisar (Haryana).

4.3.1 To study the inhibitory effect of essential oils against the bacterial isolates

The bacterial isolates were checked for their susceptibility against three essential oils i.e. Eucalyptus oil, Citronella oil and Lemon oil. The results of inhibitory effect of tested essential oils revealed in Table-4.7 and found the effect of essential oils against all bacterial isolates. The zone of inhibition was decreased as the concentration of essential oils decreases. The maximum zone of inhibition (10mm) by the eucalyptus oil with 100% concentration (0.90mg/µl) was seen in FR8 {plate no-44(b)}. Citronella oil was found most effective against HP4 {plate no-45(d)} with zone of inhibition (15mm) at 100% concentration (0.89mg/µl). HB2 {plate no-46(e)} also having maximum 14mm zone of inhibition by the lemon oil at 100% concentration (0.85mg/µl) (Table 4.7).

HSb {plate no-44(e)} and HAn {plate no-44(e)} were resistant to the eucalyptus oil with 25% concentration (0.22mg/µl). HP3 {plate no-45(e)} was resistant to the citronella oil with 25% concentration (0.22mg/µl). Similarly, both isolates FR4 {plate no-46(d)} and HB8 {plate no-46(c)} were resistant to the lemon oil with 100% (0.85mg/µl), 50% (0.42mg/µl) and 25% (0.21mg/µl) concentration (Table 4.7).

Table 4.6: Essential oils (EOs) resistance to different bacterial isolates of fresh water snail

Sr. No.	Isolate No.	Essential oil(s) (EOs)
1.	**FR4**	Lemon oil
2.	**HB8**	Lemon oil
3.	**HSb**	Eucalyptus oil
4.	**HAn**	Eucalyptus oil
5.	**HP3**	Citronella oil

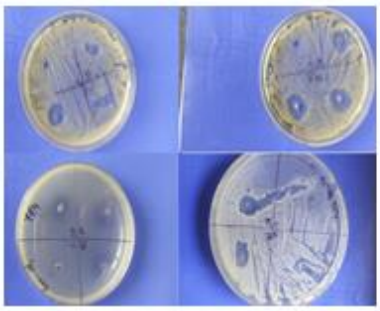


Plate 44(a): Isolate no-HP3, HP8, HP1, HP9

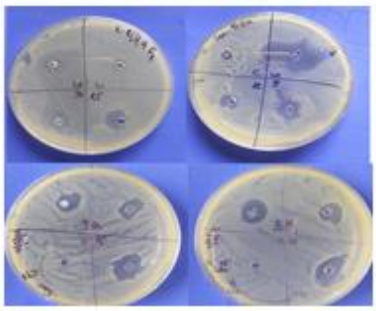


Plate 44(b): Isolate no-FR8, FR4, FR2, FR3

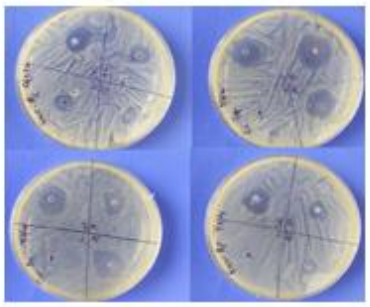


Plate 44(c): Isolate no-HB2, HB7, HB8, HB5

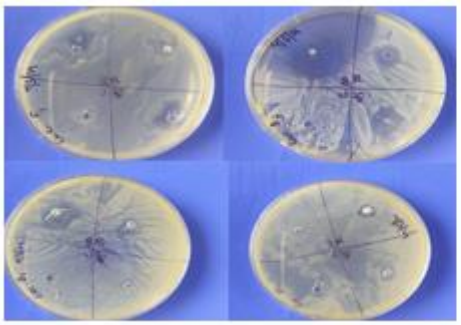


Plate 44(d): Isolate no-FR1, HB3, HB1, HAn

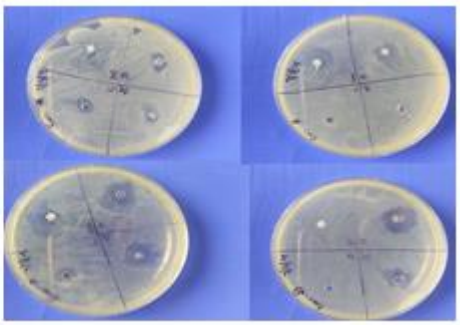


Plate 44(e): Isolate no-HSa, HSb, HSx, HAd

Plate 44(a-e): Effect of Eucalyptus oil on the bacterial isolates

Table 4.7- Comparative study of three essential oils against the bacterial isolates {Zone of inhibition (in mm)}

Isolate No.	Control (DMSO)	Eucalyptus oil			Citronella oil			Lemon oil		
		25% (0.22mg/µl)	50% (0.45mg/µl)	100% (0.90mg/µl)	25% (0.22mg/µl)	50% (0.44mg/µl)	100% (0.89mg/µl)	25% (0.21mg/µl)	50% (0.42mg/µl)	100% (0.85mg/µl)
HSa	0	3	4	5	6	7	8	9	10	**11**
HSb	0	**0**	3	4	7	9	10	8	9	10
HSx	0	4	5	6	7	9	10	5	6	7
HSj	0	3	6	7	5	6	7	5	6	7
HAd	0	6	7	8	5	6	9	9	10	**11**
HAo	0	5	6	7	8	9	10	5	6	7
HAn	0	**0**	2	3	7	8	9	4	5	6
HAf	0	4	5	6	8	9	10	6	7	8
HB1	0	3	6	9	5	6	7	4	5	6
HB2	0	3	4	5	5	8	10	12	13	**14**
HB3	0	7	8	9	5	6	7	5	6	7
HB4	0	4	5	6	7	8	**11**	3	7	8
HB5	0	3	6	8	4	4	5	9	10	**11**
HB6	0	6	7	8	7	8	9	11	12	**13**
HB7	0	5	6	7	4	5	6	9	10	**12**
HB8	0	7	8	9	7	9	**11**	**0**	**0**	**0**
HB9	0	5	6	7	6	7	8	9	10	**11**
HB10	0	4	5	6	7	8	9	4	5	6
HB11	0	4	5	6	7	8	9	3	4	5
HB12	0	6	7	8	6	7	8	8	9	**10**
FR1	0	6	7	8	3	4	5	6	7	8
FR2	0	3	5	6	5	5	6	4	5	6
FR3	0	3	4	5	3	4	5	5	7	9
FR4	0	5	6	7	5	7	9	**0**	**0**	**0**
FR5	0	5	6	7	5	7	9	8	9	**10**
FR6	0	4	5	6	7	8	9	4	5	6
FR7	0	6	7	8	6	8	10	5	6	7
FR8	0	8	9	**10**	6	8	9	9	10	**11**
HP1	0	3	5	8	7	8	10	6	7	8
HP2	0	7	8	9	6	7	8	5	6	7
HP3	0	5	6	7	**0**	5	6	7	8	9
HP4	0	4	5	6	9	12	**15**	5	6	7
HP5	0	6	7	8	8	9	10	3	5	8
HP6	0	3	3	7	5	7	8	6	7	8
HP7	0	6	7	8	6	8	9	3	4	5
HP8	0	6	7	8	5	6	7	7	8	9
HP9	0	3	4	5	6	7	8	5	6	7
HP10	0	3	4	5	8	9	10	7	8	9

0-5mm= Resistant; 5-8mm= Sensitive; 8-15mm= Most sensitive

Factors	C.D.	SE(d)	SE(m)
Factor(A)	0.526	0.268	0.19
Factor(B)	0.148	0.075	0.053
Intraction A X B	0.912	0.464	0.328
Factor(C)	0.148	0.075	0.053
Intraction A X C	N/A	0.464	0.328
Intraction B X C	N/A	0.13	0.092
Intraction A X B X C	N/A	0.804	0.569

The effect of factor A (bacterial isolates), factor B (essential oils) and factor C (concentrations) had been found significant. Two factor interaction between factor A and factor B also found significant whereas rest of the two factor and three factor interactions were insignificant.

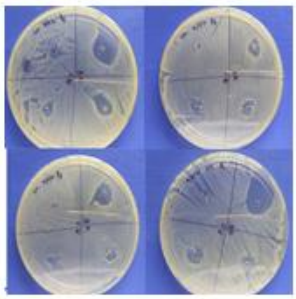


Plate 45 (a): Isolate no- HB2, HB5, HB3, HB1

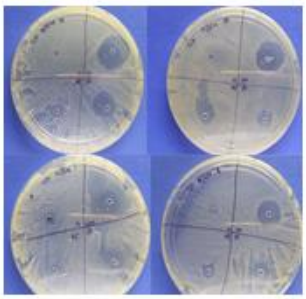


Plate 45 (b): Isolate no- HAn, HAo, HSa, HSb

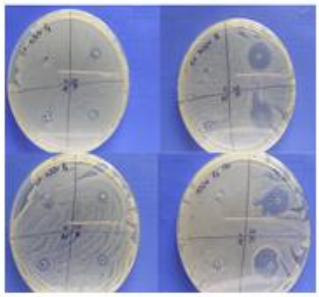


Plate 45 (c): Isolate no- FR2, FR6, FR3, FR7

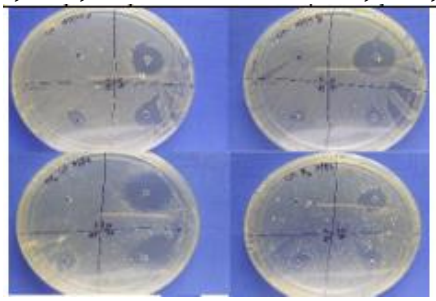


Plate 45 (d): Isolate no- HSx, HAd, HP4, HB10

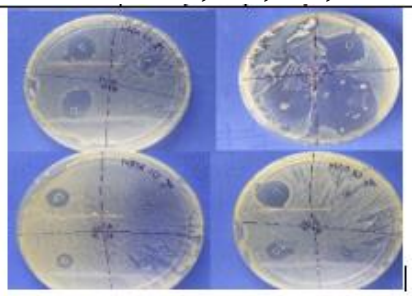


Plate 45 (e): Isolate no- HP6, HP10, HP3, HP7

Plate 45(a-e): Effect of Citronella oil on the bacterial isolates

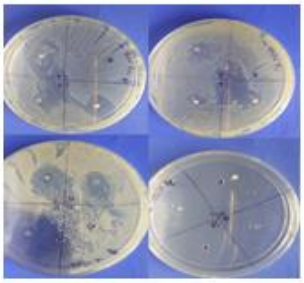


Plate 46 (a): Isolate no- HB12, HB11, HB6, HB8

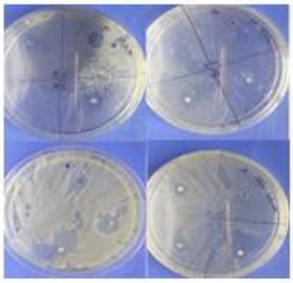


Plate 46 (b): Isolate no- HAf, HAo, HSa, HSx

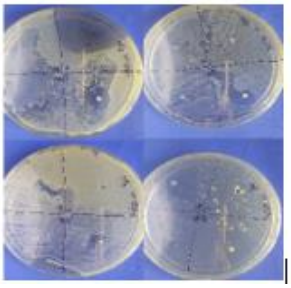


Plate 46 (c): Isolate no- HP8, HP5, HP7, HP4

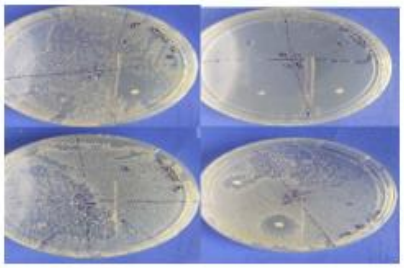


Plate 46 (d): Isolate no- FR3, FR4, FR6, FR8

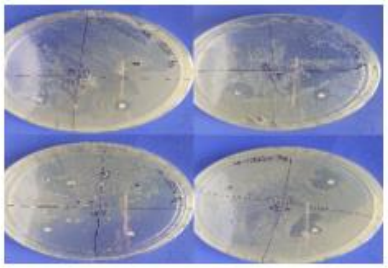


Plate 46 (e): Isolate no- HB3, HB1, HB2, HB4

Plate 46(a-e): Effect of Lemon oil on the bacterial isolates

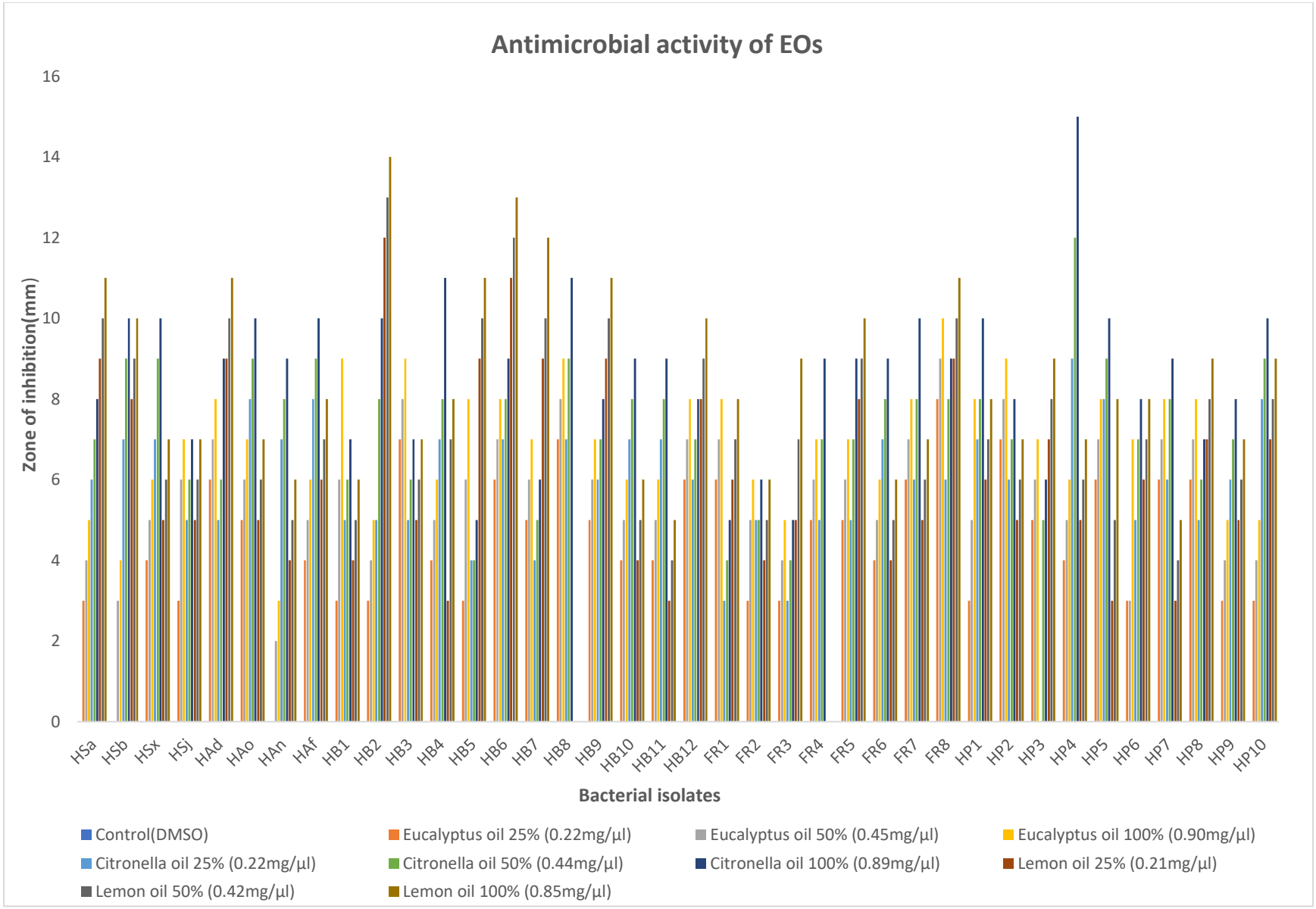


Figure 3: Inhibitory effects of essential oils against bacterial isolates

4.3.2Antibiotic well diffusion test for bacteria isolated from fresh water snail

The bacterial isolates were checked for their properties against various antibiotics after secondary test (Himedia™ chemicals). All the bacterial isolates were checked for antibiotic susceptibility test for five antibiotics. The results of inhibitory effects of antibiotics revealed in Table-4.8 and found that the effects of antibiotics were different for all bacterial isolates; none of the isolates was multidrug resistant.

The zone of inhibition was increased as the concentration of antibiotics increases. The maximum zone of inhibition (20mm) was recorded in isolate HP4 with 100% concentration of cefexime (0.04mg/µl) {plate no-47(b)}. The zone of inhibition (17mm) was maximum recorded in isolate HP2 and isolate HP4 with 100% concentration of ofloxacin (0.04mg/µl) {plate no-48(a) & (b)}. The maximum zone of inhibition was seen in isolate HB10 and isolate HP3 (18mm) with 100% concentration of chloramphenicol (0.04mg/µl) {plate no-49(e) & (b)}. The zone of inhibition (20mm) was maximum seen in isolate HP2 with 100% concentration of ampicillin (0.04mg/µl) {plate no-50(d)}. The maximum zone of inhibition (14mm) was seen in isolate HP2 with 100% concentration of rifampin + isoniazid (0.04+0.026mg/ µl) {plate no-51(b)} (Table 4.8).

From the Table-4.8 it was found that isolates HSb, HP6 and HP9 {plate no-47(d), (c)} were resistant with 25% concentration against cefexime (0.01mg/µl); HSa, HAn, FR6, FR8 and HB9 {plate no-50(d), (c) & (b)} were resistant with 25% concentration against ampicillin (0.01mg/µl) and HB7 were resistant with 25% concentration against rifampin + isoniazid (0.01+0.006mg/µl) {plate no-51(a)}.

Table 4.8: Comparative study of five antibiotics against the bacterial isolates {Zone of inhibition (in mm)}

Isolate	Control	Cefexime			Ofloxacin			Chloramphenicol			Ampicillin			Rifampin+Isoniazid		
No.	(DDW)	25%	50%	100 %	25%	50%	100 %	25%	50%	100 %	25%	50%	100 %	25%	50%	100%
		(0.01mg/µl)	(0.02mg/µl)	(0.04mg/µl)	(0.01 mg/µl	(0.02mg/µl)	(0.04mg/µl)	(0.01mg/µl)	(0.02mg/µl)	(0.04mg/µl)	(0.01mg/µl)	(0.02mg/µl)	(0.04mg/µl)	(0.01+0.006 mg)/µl	(0.02+0.012 mg)/µl	(0.04+0.026 mg)/ µl
HSa	0	3	4	5	4	3	6	12	13	14	0	5	6	8	9	10
HSb	0	0	9	10	6	2	8	12	11	12	8	9	10	6	9	10
HSx	0	11	13	14	11	12	14	10	13	14	8	9	10	5	8	9
HSj	0	9	10	11	11	12	13	12	11	13	7	8	9	8	9	10
HAd	0	10	11	12	10	10	9	10	8	9	9	10	11	6	7	8
HAo	0	12	14	16	12	12	13	7	10	11	9	10	11	8	9	9
HAn	0	10	11	12	11	12	13	8	11	15	0	4	5	10	11	12
HAf	0	8	10	11	11	12	13	9	12	13	9	10	11	8	9	10
HB1	0	7	9	8	8	8	9	11	14	15	17	18	**19**	7	8	9
HB2	0	10	11	12	6	8	9	13	16	17	14	15	16	10	11	12
HB3	0	14	15	16	8	9	10	15	14	15	14	15	16	10	11	12
HB4	0	10	11	12	6	7	8	13	15	16	13	14	15	11	12	13
HB5	0	11	12	13	9	10	11	14	15	16	10	12	14	6	9	10
HB6	0	3	6	7	6	8	9	14	11	12	8	9	10	3	9	10
HB7	0	13	14	15	8	10	11	13	14	15	14	15	16	0	8	10
HB8	0	12	13	14	8	9	10	9	10	11	13	14	15	11	12	13
HB9	0	5	7	8	6	8	9	9	12	14	0	4	5	7	8	9
HB10	0	10	11	12	6	7	8	13	15	**18**	13	14	15	7	8	9
HB11	0	11	12	13	12	13	14	12	13	15	14	15	16	9	10	11
HB12	0	5	6	7	7	8	9	12	13	15	8	9	12	6	8	9
FR1	0	3	8	11	12	13	**16**	9	10	11	9	11	12	10	11	12
FR2	0	9	11	12	11	12	12	10	11	12	4	5	6	9	10	11
FR3	0	7	9	10	14	13	**16**	10	12	13	14	15	16	8	9	10
FR4	0	6	8	9	12	13	14	9	12	14	8	9	10	7	9	10
FR5	0	9	11	12	10	11	12	9	10	11	9	10	11	7	8	9
FR6	0	7	10	11	12	13	14	14	15	16	0	4	5	9	10	11
FR7	0	9	11	12	12	12	13	11	12	13	6	7	8	7	8	9
FR8	0	12	14	15	11	12	13	8	9	10	0	3	4	7	8	9
HP1	0	13	15	16	11	12	13	13	15	14	8	9	10	11	12	13
HP2	0	12	13	14	15	16	**17**	15	16	17	18	19	**20**	12	13	**14**
HP3	0	12	13	14	14	15	16	16	17	**18**	5	6	7	8	9	10
HP4	0	18	19	**20**	16	16	**17**	12	13	14	8	9	10	10	11	12
HP5	0	12	13	14	11	12	13	14	12	16	5	6	7	8	9	10
HP6	0	0	6	7	12	13	14	12	13	14	9	11	12	8	9	10
HP7	0	15	16	17	12	13	14	15	16	17	7	8	9	6	8	9
HP8	0	9	13	14	11	12	13	11	12	13	5	7	6	8	9	10
HP9	0	0	3	4	13	14	15	12	13	14	5	5	6	8	9	10
HP10	0	15	16	17	12	12	13	13	14	15	4	5	6	10	11	12

Factors	C.D.	SE(d)	SE(m)
Factor(A)	0.406	0.207	0.146
Factor(B)	0.147	0.075	0.053
Intraction A X B	0.907	0.462	0.327
Factor(C)	0.114	0.058	0.041
Intraction A X C	0.703	0.358	0.253
Intraction B X C	0.255	0.13	0.092
Intraction A X B X C	1.571	0.801	0.566

The main effect of factor A (bacterial isolates), factor B (antibiotics), and factor C (concentrations) had been found significant. The two factor intraction between factor A and factor B found significant whereas the rest of the two factor and three factor intractions were also found significant.

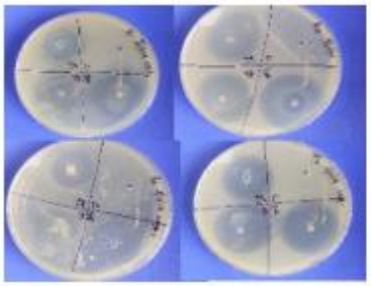


Plate 47 (a): Isolate no- HB5, HB11, HB7, HP2

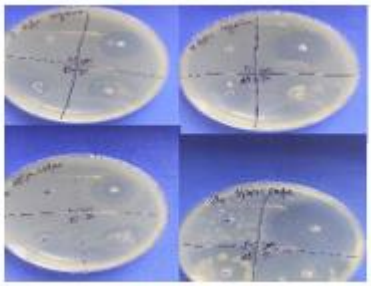


Plate 47 (b): Isolate no- HSj, HSx, FR8, HP4

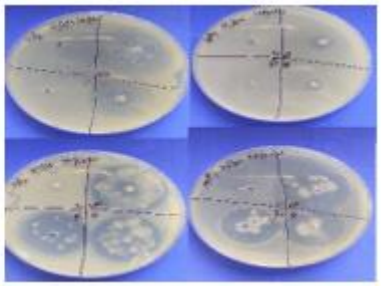


Plate 47 (c): Isolate no- HP6, HP9, HP10, HP7

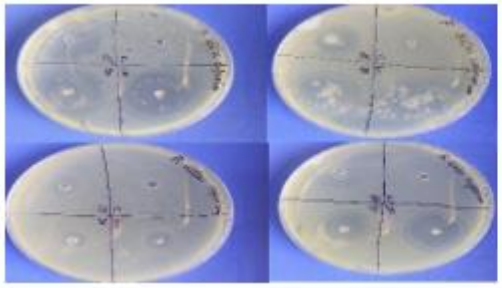


Plate 47 (d): Isolate no- HAn, HAf, HSa, HSb

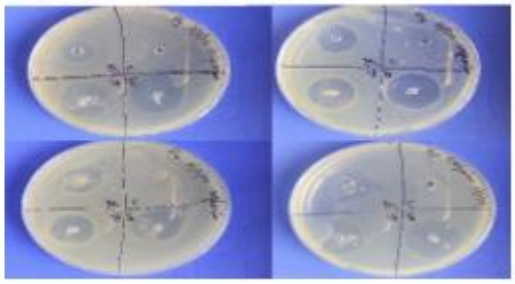


Plate 47 (e): Isolate no- FR3, FR2, FR4, FR5

Plate 47(a-e): Effect of Cefexime antibiotic on the bacterial isolates

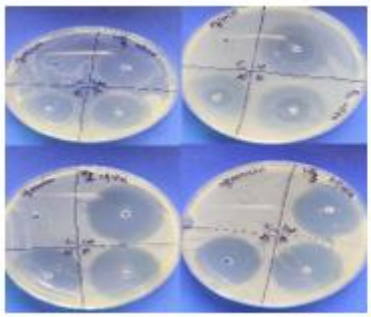


Plate 48 (a): Isolate no- HP5, HB12, HP2, HP3

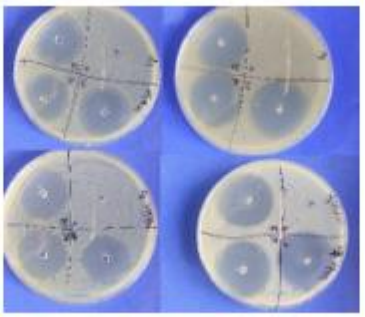


Plate 48 (b): Isolate no- HP6, FR3, FR6, HP4

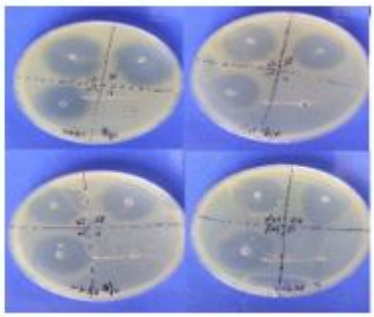
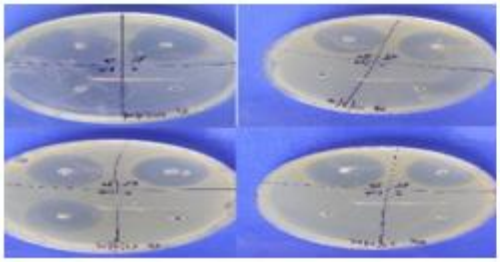


Plate 48 (c): Isolate no- HP7, HP8, HP10, HSx



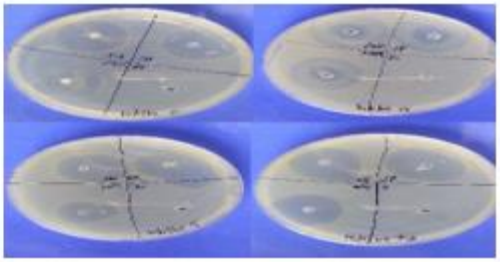
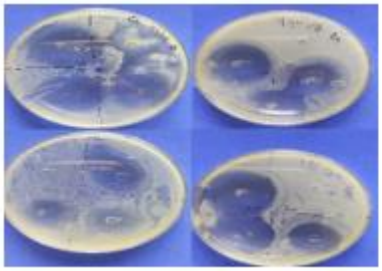
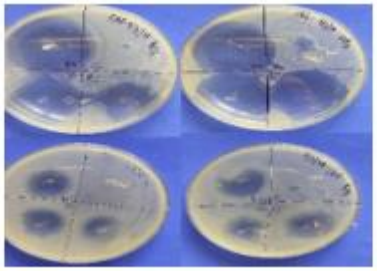


Plate 48 (d): Isolate no- FR1, FR8, FR2, FR4 **Plate 48 (e): Isolate no- HSj, HAn, HAd, FR7**

Plate 48(a-e): Effect of Ofloxacin antibiotic on the bacterial isolate





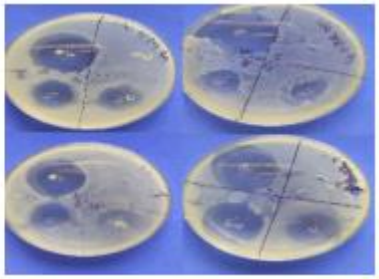
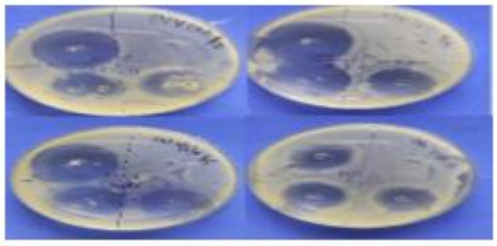


Plate 49(a): Isolate no- HSa, HB1, FR1, HB2 **Plate 49(b): Isolate no- HB11, HP3, HAd, HB8** **Plate 49(c): Isolate no- HB9, FR3, HSj, HP7**



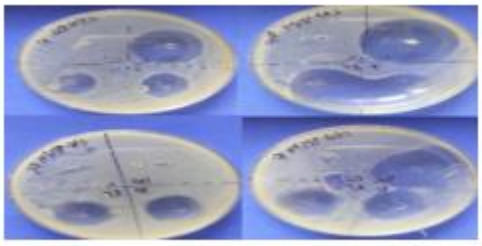
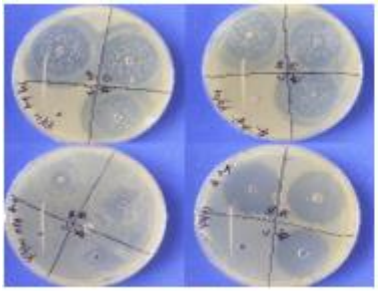
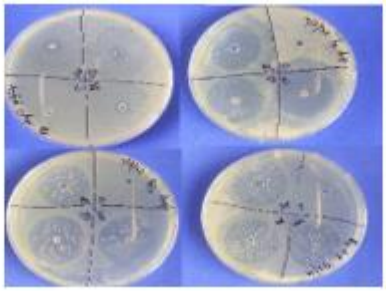


Plate 49(d): Isolate no- HB7, HB2, HB5, HSb **Plate 49(e): Isolate no- HB10, HAn, HAf, FR5**

Plate 49(a-e): Effect of Chloramphenicol antibiotic on the bacterial isolates





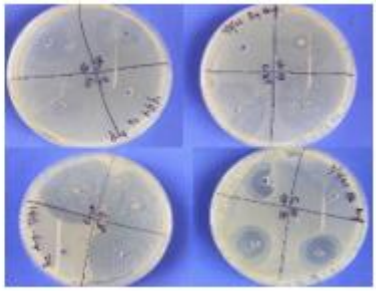


Plate 50 (a): Isolate no- HB4, HB5, HP8, HB7 **Plate 50 (b): Isolate no- FR8, HB11, HB10, HB8** **Plate 50 (c): Isolate no- FR6, HB9, HB12, HB6**

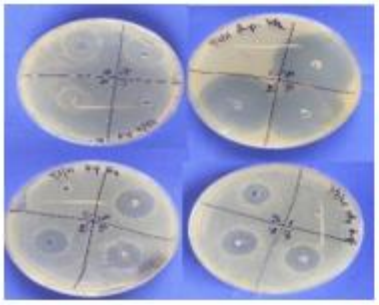


Plate 50 (d): Isolate no- HSa, HP2, FR4, HP7

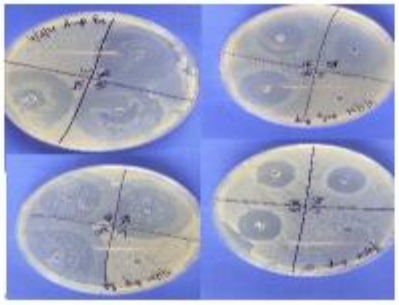


Plate 50 (e): Isolate no- HB1, HP6, HB2, HAo

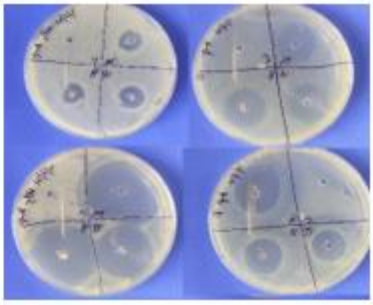


Plate 50 (f): Isolate no- HP9, HAd, HP5, HAf

Plate 50(a-f): Effect of Ampicillin antibiotic on the bacterial isolates

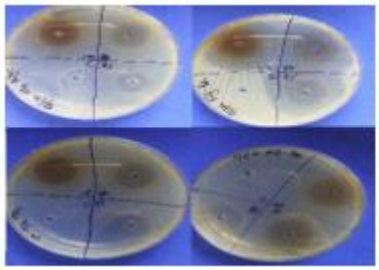


Plate 51 (a): Isolate no- HB7, HB2, HSj, HB10

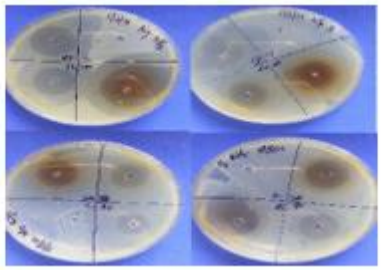


Plate 51 (b): Isolate no- HP2, HSb, HP7, FR6

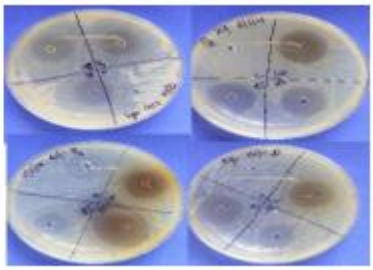


Plate 51 (c): Isolate no- HP5, FR2, FR4, HAd

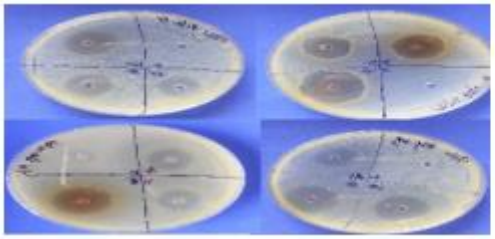


Plate 51 (d): Isolate no- HSa, HAo, HP3, HP6

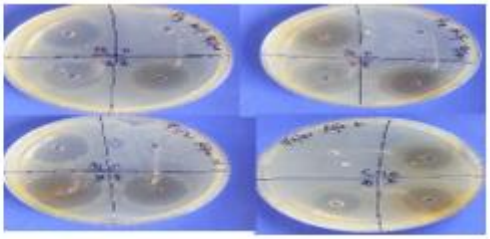


Plate 51 (e): Isolate no- FR3, FR8, HAn, HAf

Plate 51(a-f): Effect of Rifampin +Isoniazid antibiotic on the bacterial isolates

Comparative efficacy of antibiotics with the essential oils

We were applied the one-way ANOVA and T-test to check the significant difference between the antibiotics and essential oils.

Table 9- Comparative efficacy of Eucalyptus oil & Cefexime

Character	Group	N	Mean (Zone of inhibition)	Variance	Stand Dev.	Stand. Error
Character1	Eucalyptus oil	38	10.67	13.42	3.66	0.59
	Cefexime	38	5.65	2.4	1.55	0.25

T-test

Character	Group	Assumptions	T	d.f.	Probability
Character 1	Eucalyptus oil & Cefexime	Equal Variances Assumed	7.77	74	0
		Unequal Variances Assumed	7.77	49.84	0

T-Test shows that the effect of Eucalyptus oil on bacterial isolates were different from the effect of Cefexime. Both are significant from each other. Here ($p<0.05$), both character are significant from each other.

Table 10- Comparative efficacy of Eucalyptus oil & Ofloxacin

Character	Group	N	Mean (Zone of inhibition)	Variance	Stand Dev.	Stand. Error
Character1	Eucalyptus oil	38	10.74	8.69	2.95	0.48
	Ofloxacin	38	5.65	2.4	1.55	0.25

T-test

Character	Group	Assumptions	T	d.f.	Probability
Character 1	Eucalyptus oil& Ofloxacin	Equal Variances Assumed	9.4	74	0
		Unequal Variances Assumed	9.4	56	0

T-Test shows that the effect of Eucalyptus oil on bacterial isolates were different from the effect of Ofloxacin. Both are significant from each other. Here ($p<0.05$), both character are significant from each other.

Table 11- Comparative efficacy of Eucalyptus oil & Chloramphenicol

Character	Group	N	Mean (Zone of inhibition)	Variance	Stand Dev.	Stand. Error
Character1	Eucalyptus oil	38	12.58	5.97	2.44	0.4
	Chloramphenicol	38	5.65	2.4	1.55	0.25

T-test

Character	Group	Assumptions	T	d.f.	Probability
Character1	Eucalyptus oil & Chloramphenicol	Equal Variances Assumed	14.74	74	0
		Unequal Variances Assumed	14.74	62.63	0

T-Test shows that the effect of Eucalyptus oil on bacterial isolates were different from the effect of Chloramphenicol. Both are significant from each other. Here ($p<0.05$), both character are significant from each other.

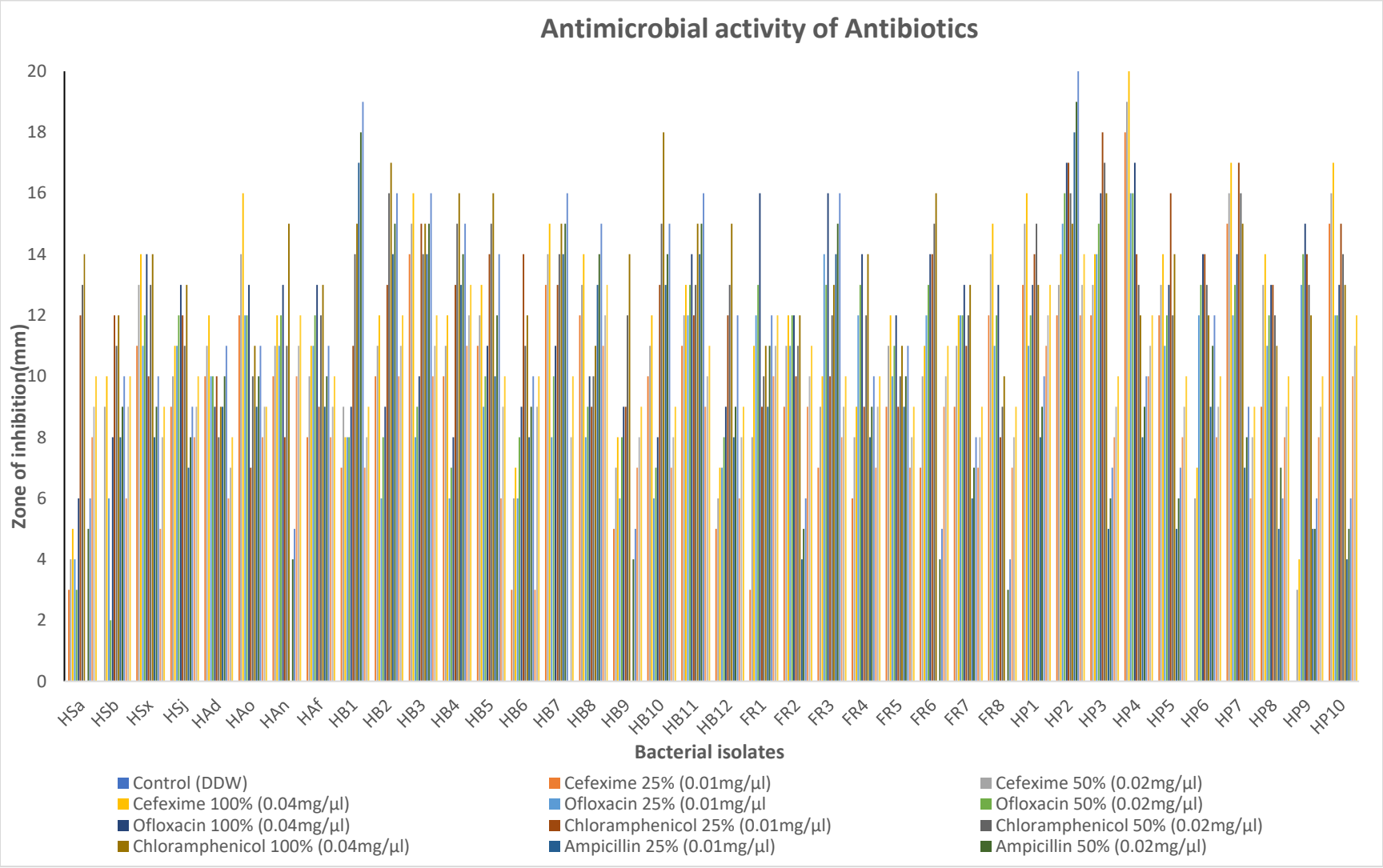


Figure 4: Inhibitory effects of antibiotics against bacterial isolates

Table 12- Comparative efficacy of Eucalyptus oil & Ampicillin

Character	Group	N	Mean (Zone of inhibition)	Variance	Stand Dev.	Stand. Error
Character1	Eucalyptus oil	38	9.51	19.06	4.37	0.71
	Ampicillin	38	5.65	2.4	1.55	0.25

T-test

Character	Group	Assumptions	T	d.f.	Probability
Character1	Eucalyptus oil & Ampicillin	Equal Variances Assumed	5.13	74	0
		Unequal Variances Assumed	5.13	46.18	0

T-Test shows that the effect of Eucalyptus oil on bacterial isolates were different from the effect of Ampicillin. Both are significant from each other. Here ($p<0.05$), both character are significant from each other.

Table 13- Comparative efficacy of Eucalyptus oil & Rifampin+ Isoniazid

Character	Group	N	Mean (Zone of inhibition)	Variance	Stand Dev.	Stand. Error
Character1	Eucalyptus oil	38	9.23	2.63	1.62	0.26
	Rifampin+ Isoniazid	38	5.65	2.4	1.55	0.25

T-test

Character	Group	Assumptions	T	d.f.	Probability
Character1	Eucalyptus oil & Rifampin+Isoniazid	Equal Variances Assumed	9.83	74	0
		Unequal Variances Assumed	9.83	73.85	0

T-Test shows that the effect of Eucalyptus oil on bacterial isolates were different from the effect of Rifampin+Isoniazid. Both are significant from each other. Here ($p<0.05$), both character are significant from each other.

Table 14- Comparative efficacy of Citronella oil & Cefexime

Character	Group	N	Mean (Zone of inhibition)	Variance	Stand Dev.	Stand. Error
Character1	Citronella oil	38	10.67	13.42	3.66	0.59
	Cefexime	38	7.25	2.79	1.67	0.27

T-test

Character	Group	Assumptions	T	d.f.	Probability
Character1	Citronella oil & Cefexime	Equal Variances Assumed	5.23	74	0
		Unequal Variances Assumed	5.23	51.74	0

T-Test shows that the effect of Citronella oil on bacterial isolates were different from the effect of Cefexime. Both are significant from each other. Here ($p<0.05$), both character are significant from each other.

Table15- Comparative efficacy of Citronella oil & Ofloxacin

Character	Group	N	Mean (Zone of inhibition)	Variance	Stand Dev.	Stand. Error
Character1	Citronella oil	38	10.74	8.69	2.95	0.48
	Ofloxacin	38	7.25	2.79	1.67	0.27

T-test

Character	Group	Assumptions	T	d.f.	Probability
Character 1	Citronella oil & Ofloxacin	Equal Variances Assumed	6.34	74	0
		Unequal Variances Assumed	6.34	58.52	0

T-Test shows that the effect of Citronella oil on bacterial isolates were different from the effect of Ofloxacin. Both are significant from each other. Here ($p<0.05$), both character are significant from each other.

Table 16- Comparative efficacy of Citronella oil &Chloramphenicol

Character	Group	N	Mean (Zone of inhibition)	Variance	Stand Dev.	Stand. Error
Character1	Citronella oil	38	12.58	5.97	2.44	0.4
	Chloramphenicol	38	7.25	2.79	1.67	0.27

T-test

Character	Group	Assumptions	T	d.f.	Probability
Character1	Citronella oil & Chloramphenicol	Equal Variances Assumed	11.08	74	0
		Unequal Variances Assumed	11.08	65.37	0

T-Test shows that the effect of Citronella oil on bacterial isolates were different from the effect of Chloramphenicol. Both are significant from each other. Here ($p<0.05$), both character are significant from each other.

Table 17- Comparative efficacy of Citronella oil & Ampicillin

Character	Group	N	Mean (Zone of inhibition)	Variance	Stand Dev.	Stand. Error
Character1	Citronella oil	38	9.51	19.06	4.37	0.71
	Ampicillin	38	7.25	2.79	1.67	0.27

T-test

Character	Group	Assumptions	T	d.f.	Probability
Character1	Citronella oil & Ampicillin	Equal Variances Assumed	2.98	74	0.0039
		Unequal Variances Assumed	2.98	47.6	0.0046

T-Test shows that the effect of Citronella oil on bacterial isolates were different from the effect of Ampicillin. Both are significant from each other. Here ($p<0.05$), both character are significant from each other.

Table 18- Comparative efficacy of Citronella oil & Rifampin+ Isoniazid

Character	Group	N	Mean (Zone of inhibition)	Variance	Stand Dev.	Stand. Error
Character1	Citronella oil	38	9.23	2.63	1.62	0.26
	Rifampin+ Isoniazid	38	7.25	2.79	1.67	0.27

T-test

Character	Group	Assumptions	T	d.f.	Probability
Character1	Citronella oil & Rifampin+Isoniazid	Equal Variances Assumed	5.24	74	0
		Unequal Variances Assumed	5.24	73.94	0

T-Test shows that the effect of Citronella oil on bacterial isolates were different from the effect of Rifampin+Isoniazid. Both are significant from each other. Here ($p<0.05$), both character are significant from each other.

Table 19- Comparative efficacy of Lemon oil & Cefexime

Character	Group	N	Mean (Zone of inhibition)	Variance	Stand Dev.	Stand. Error
Character1	Lemon oil	38	10.67	13.42	3.66	0.59
	Cefexime	38	6.93	7.53	2.74	0.45

T-test

Character	Group	Assumptions	T	d.f.	Probability
Characte1	Lemon oil& Cefexime	Equal Variances Assumed	5.03	74	0
		Unequal Variances Assumed	5.03	68.57	0

T-Test shows that the effect of Lemon oil on bacterial isolates were different from the effect of Cefexime. Both are significant from each other. Here ($p<0.05$), both character are significant from each other.

Table 20- Comparative efficacy of Lemon oil & Ofloxacin

Character	Group	N	Mean (Zone of inhibition)	Variance	Stand Dev.	Stand. Error
Character1	Lemon oil	38	10.74	8.69	2.95	0.48
	Ofloxacin	38	6.93	7.53	2.74	0.45

T-test

Character	Group	Assumptions	T	d.f.	Probability
Character 1	Lemon oil& Ofloxacin	Equal Variances Assumed	5.82	74	0
		Unequal Variances Assumed	5.82	73.62	0

T-Test shows that the effect of Lemon oil on bacterial isolates were different from the effect of Ofloxacin. Both are significant from each other. Here ($p<0.05$), both character are significant from each other.

Table 21- Comparative efficacy of Lemon oil &Chloramphenicol

Character	Group	N	Mean (Zone of inhibition)	Variance	Stand Dev.	Stand. Error
Character1	Lemon oil	38	12.58	5.97	2.44	0.4
	Chloramphenicol	38	6.93	7.53	2.74	0.45

T-test

Character	Group	Assumptions	T	d.f.	Probability
Character1	Lemon oil & Chloramphenicol	Equal Variances Assumed	9.47	74	0
		Unequal Variances Assumed	9.47	73.03	0

T-Test shows that the effect of Lemon oil on bacterial isolates were different from the effect of Chloramphenicol. Both are significant from each other. Here ($p<0.05$), both character are significant from each other.

Table 22- Comparative efficacy of Lemon oil & Ampicillin

Character	Group	N	Mean (Zone of inhibition)	Variance	Stand Dev.	Stand. Error
Character1	Lemon oil	38	9.51	19.06	4.37	0.71
	Ampicillin	38	6.93	7.53	2.74	0.45

T-test

Character	Group	Assumptions	T	d.f.	Probability
Character1	Lemon oil & Ampicillin	Equal Variances Assumed	3.08	74	0.0029
		Unequal Variances Assumed	3.08	62.27	0.0031

T-Test shows that the effect of Lemon oil on bacterial isolates were different from the effect of Ampicillin. Both are significant from each other. Here ($p<0.05$), both character are significant from each other.

Table 23- Comparative efficacy of Lemon oil & Rifampin+ Isoniazid

Character	Group	N	Mean (Zone of inhibition)	Variance	Stand Dev.	Stand. Error
Character1	Lemon oil	38	9.23	2.63	1.62	0.26
	Rifampin+ Isoniazid	38	6.93	7.53	2.74	0.45

T-test

Character	Group	Assumptions	T	d.f.	Probability
Character1	Lemon oil & Rifampin+Isoniazid	Equal Variances Assumed	4.45	74	0
		Unequal Variances Assumed	4.45	60.04	0

T-Test shows that the effect of Lemon oil on bacterial isolates were different from the effect of Rifampin+Isoniazid. Both are significant from each other. Here ($p<0.05$), both character are significant from each other.

Table 24- Comparative efficacy of all EOs & all antibiotics

Character	Group	N	Mean (Zone of inhibition)	Variance	Stand Dev.	Stand. Error
Character1	all Eos	114	6.61	4.65	2.16	0.2
	all antibiotics	190	10.54	11.15	3.34	0.24

T-test

Character	Group	Assumptions	T	d.f.	Probability
Character1	all EOs & all antibiotics	Equal Variances Assumed	11.24	302	0
		Unequal Variances Assumed	12.47	300.36	0

T-Test shows that the effects of all EOs on bacterial isolates were different from the effect of all Antibiotics. Both are significant from each other. Here ($p<0.05$), both character are significant from each other.

CHAPTER-V

DISCUSSION

Aquatic molluscs are supreme invertebrate model systems for toxicology and environmental monitoring (Rittschof and McClellan-Green, 2005). Freshwater molluscs entitle the ecologists to judge the health of their habitat and help in the ecosystem's functioning (Kumar *et al*. 2019). Altindag and Yigit (2005) reported that gastropods are used as food source by birds and fishes, which in turn make them reachable for human consumption through food chain and in due course pose great health risk.

Snails are of great concern in agriculture, medical and veterinary practices due to damage caused by them to agriculture, horticulture and forestry as well as their main role as intermediate hosts for the trematodes causing schistosomiasis and fascioliasis in humans and domestic animals. Molluscs plays an important role in livelihood and economy (Borkakati *et al.* 2009)

They are abundant in the rainy season, but undergo aestivation and hibernation, during the dry and winter seasons respectively, by forming a membrane over the shell opening to reduce water loss (Amusan and Omidijii, 1999). Hamzat (2003) indicated that snails are well adapted to adverse environmental conditions, such as temperature, heat and cold fluctuation and they have natural immunity against disease causing organisms, such as *Staphylococcus, Streptococcus* and other bacterial species. Snails have high fecundity and survival (Akinbile, 2000).

In African communities they are available as a source of nutrition and nourishment (Meyer-Rochow, 2009). Snails are recognised as 'Congo meat' as they are highly delicious (Fagbuaro *et al*. 2006). Ghosh *et al.* (2016) observed that gastropods are the source of nutrition comparable to or even superior to traditional livestock as snail protein is a potential option beside animal protein in term of choice. Ademolu *et al.* (2004) and Etchu *et al.* (2008) reported that snail meat possesses Iron and is also cholesterol free and being appetizing has become a choice in diet, among the meat eaters. Among minerals, Calcium is the most abundant of all followed by Phosphorous, Iron and Zinc (Ghosh *et al.* 2017). Some edible species of fresh water snails are *Anisus convexiusculus, B. bengalensis, L. marginalis, M. tuberculata* and *P. globosa* (Baby *et al.* 2010). Snail meat act as neutralizer and antidote in people suffering from vascular diseases like heart attack and hypertension (Iheke and Nwankwo, 2016).

The aqueous extract of latex of *E. pulcherrima, T. peruviana* and *A. scholaris* caused mortality against two harmful freshwater snails i.e. *L. acuminata* and *I. exustus* (Singh & Singh, 2005). Lach *et al.* (2000) reported that the freshwater apple snail *P. canaliculata* has become a major crop pest in southeast Asia and Hawaii and threatens natural wetland habitats.

Snail facilitates the spread of a potentially fatal human brain disease (Eosinophilic meningitis) caused by the nematode *Angiostrongylus cantonensis* (Tsai *et al.* 2004; Lv *et al.* 2008). They can directly transmit disease or serve as intermediate hosts for parasites of humans and animals (Pearce & MacDonald, 2002; Sokolow *et al.* 2016). Morley in (2010) reported that sometimes they act only as

'mechanical vectors' rather than genuine hosts as cysts of human diseases, such as *Cryptosporidium* and *Giardia* are found in filter feeding molluscs.

For snails, differences in population dynamics among habitats can be driven by a variety of factors including differences in food quantity, quality & availability, water velocities, water depth, waterbody size, abundance of competitors, the diversity and abundance of predators or parasites, or other factors that affect survivorship and reproduction (Burlakova *et al.* 2010).

Molluscs are also among the most intractable of pests (Barker and Watts, 2002). The pest gastropods not only directly damage the agricultural crops in the field but also lower the quality by soiling with slime and faeces. The snail affected portions of agricultural product are contaminated by rotting agents such as bacteria and fungi, which lead to further damage of fruits and vegetables in storage.

Adebayo-Tayo *et al*. (2011) assessed the microbiological content of freshwater snail samples and revealed that the total microbial diversity were *Proteus, Escherichia, Klebsiella, Streptococcus, Pseudomonas, Shigella, Serratia, Vibrio, Actinectobacter, Yersinia, Providencia, Mycobacterium, Enterobacter, Bacillus, Micrococcus, Staphylococcus, Citrobacter, Samonella* and *Nesseria* whereas fungal isolates were *Geotrichum, Botrytis, Cladosporium, Curvularia, Fusarium, Mucor, Aspergillus, Neurospora, Penicillium, Helminthosporium, Trichodema* and *Cryptomonas* (Ehigiator *et al*. 2015).

Donkor *et al.* (2017) reported that haemolymph of *A. achatina* contains multi-drug resistant isolates of *S. aureus, P. aeruginosa* & *E. coli* and these pathogens were the leading causes of infections in Ghana (Opintan *et al.* 2015; Newman *et al*. 2011). *S. aureus* was responsible for a wide range of invasive infections including septicaemia, pneumonia, endocarditis, meningitis and osteomyelitis (Todar, 2006; Schaumburg *et al.* 2014). *P. aeruginosa* known to cause several types of infections particularly in immune-compromised individuals (Lila *et al.* 2017). *E. coli* was the leading cause of urinary tract infections (Alos, 2005) and also implicated in several other diseases particularly diarrhea (Abba *et al.* 2009).

To isolate and characterize the bacterial isolates retrieved from fresh water snails

Heliciculture or Molluscan aquaculture is invaded by infectious diseases which are a major hindrance to its development and are one of the most significant causes of ecological and economic losses. These infectious diseases are caused by the interactive effects of viral, bacterial, protozoan and trematode infections with toxic pollutants (Morley, 2010). One of the most common mycobacterial diseases of humans is chronic skin ulcers (i.e. Buruli ulcer disease) caused by the *M. ulcerans* infections from the tropical aquatic *P. canaliculata* (Ampullariidae) and *Planorbis planorbis* (Planorbidae) snails (Marsollier *et al.* 2004).

In the present study the visceral mass or tissue of fresh water snail was taken for the isolation of bacterial isolates which was highly successful sampling method. In our study thirty-eight bacterial isolates were isolated from the snail samples of five different localities and studied in Rodentology

Laboratory, Department of Zoology & Aquaculture, CCSHAU, Hisar. Out of them, twenty bacterial isolates were Gram negative and eighteen were Gram positive. The shape of isolates was varying from rod/bacillus to cocci. On the basis of various secondary biochemical tests, these isolates belong to *Staphylococcus, Neisseria, Yersinia, Micrococcus, Clostridium, Enterobacter, Campylobacter, Pseudomonas, Proteus, Streptococcus, Enterococcus, Bacillus, Salmonella* and *Aeromonas* genera in collected snail samples.

Intestinal microorganisms are important for activity of hydrobionts. Shtykova *et al.* (2018) studied the microbiota composition of endemic gastropods *Benedictia baicalensis* (Caenogastropoda) and of the bottom sediments and concluded that isolated bacteria belonged to eleven genera of heterotrophic bacteria, such as *Pseudomonas, Acinetobacter, Alcaligenes, Flavobacterium, Enterobacter, Escherichia, Citrobacter, Klebsiella, Bacillus, Staphylococcus, Streptococcus* and *Micrococcus.* It was also revealed that about 95–97% strains were found Gram-negative bacteria in *B. baicalensis* and in the bottom sediments 51% of the isolates were Gram-positive coccoid and spore-forming rod-shaped bacteria. Similary, in our finding there is greater proportion of Gram-negative bacterial isolates and also some bacterial isolates were Gram-positive coccoid *i.e. Staphylococcus, Streptococcus* and *Micrococcus* and spore-forming rod-shaped bacteria, such as *Clostridium* and *Bacillus*.

Similarly, Marsollier *et al.* (2004) also found the pathogens, such as *Salmonella, Clostridium Botulinum, Listeria* and *Mycoplasmas* were present in the mollusc intestines in his studies. Bassey *et al.* (2014) identified *S. epidermidis, S. aureus, S. pneumonia, S. pyrogens, E. coli, Micrococcus* and *Serrata marcesseus* from the *Pomecia palludus.*

Barlett (1976) identified 108 viable mesophilic bacteria belonged to sixteen genera of bacteria in which Gram negatives were predominating. These bacterial isolates were *Salmonella, Aeromonas, P. aeruginosa, Acinetobacter, Chromobacter, Alcaligenes, Citrobacter, Clostridium, Proteus, Bacillus, Edwardsiella, Escherichia, Enterobacter, Streptococcus, Flavobacterium* and *Vibrio.* Similarly, *Clostridium, Proteus, Bacillus, Salmonella, Enterobacter, Streptococcus, Aeromonas* and *Pseudomonas* were isolated and identified in our research findings.

Van Horn *et al.* (2012) investigated intestinal microbiota of three species of snails across the phylogenetic range of Planorbidae: *Bulinus africanus* (a basal planorbid), *Biomphalaria pfeifferi* and *Helisoma duryi* and characterized the diversity of bacterial using 16S rRNA gene sequences within the intestines and identified the bacterial diversity of Acidobacteria, Bacteroidetes and Gammaproteobacteria.

Ugoh and Ugbenyo (2013) revealed that the total aerobic bacteria and other organisms of public health importance were *Salmonella, coliforms, Vibrio, Shigella* and *Staphylococcus* from the aerobic bacterial population associated with freshwater apple snail (*P. ampullacea*) and its environment (water). Similarly, Opara & Okpe (2019) reported that the bacteria which predominated the snail flora were

Vibrio, Aeromonas, Pseudomonas, Acinetobcter and several others members of family Enterobacteriaceae.

Evaluation of antimicrobial activity of essential oils against bacterial isolates

Medicinal plants are important source for the verification of pharmacological effects and also natural composite sources that act as new anti-infectious agents (Ushimaru *et al.* 2007). Historically, many plant oils and extracts, such as tea tree, myrrh and clove, used as topical antiseptics, or reported to have antimicrobial properties.

In this study, the antibacterial activity of Essential oils (EOs) was investigated against the bacteria isolated from fresh water snail samples collected from water bodies or ponds from five different localities. The isolate HSb was found resistant with eucalyptus oil at 25% concentration (0.22mg/µl) but sensitive to 50% (0.45mg/µl) and 100% (0.90mg/µl) concentrations. Similarly, isolate HAn found resistant with eucalyptus oil at 25% concentration (0.22mg/µl) but sensitive to 50% and 100% concentrations. HP3 was found resistant to the citronella oil with 25% concentration (0.22mg/µl) but susceptible to 50% (0.44mg/µl) and 100% (0.89mg/µl) concentrations. Both isolates FR4 and HB8 were found resistant with lemon oil at all three concentrations i.e. 25% (0.21mg/µl), 50% (0.42mg/µl) and 100% (0.85mg/µl).

Ajay (2018) reported that zone of inhibition increased whenever the concentration of essential oil increased. In accordance with this, similar results were found in our study i.e. size of zone of inhibition was increased with increase in concentration. Lemon oil was found most effective against all the bacterial isolates.

Indrayan *et al.* (2011) studied the antibacterial activity of essential oil from the rhizome of *Canna indica* against three micro-organisms i.e. *E. coli., S. aureus* and *B. subtilis*. Results showed good antibacterial activity against *S. aureus*, mild against *B. subtilis* and nil against *E. coli*.

Antimicrobial activity of plant extracts i.e. apricot kernel, evening primrose, macadamia, pumpkin, sage, sweet almond and other essential oils i.e. Lemon grass, oregano, bay leaves were investigated for activity against *S. aureus, A. veronii, Candida albicans, Klebsiella pneumoniae, Enterococcus faecalis, E. coli, P. aeruginosa, S. enterica subsp. enterica serotype typhimurium, Serratia marcescens* and *Acinetobacter baumanii* (Hammer *et al*. 1999).

Deepak (2019) reported that all the essential oils were effective against all bacteria isolated from avian fecal matter except neem seed oil. Similarly results of our study showed that all bacterial isolates were found susceptible to all three oils except isolates HB8 and FR4 were found resistance against Lemon oil.

Fico *et al*. (2004) studied the medicinal plants and seeds such as *N. damascene* and obtained various extracts, essential oil at different polarity, fractions and pure compounds for biological activity against Gram negative bacteria *E. coli, P. aeruginosa, Yersinia enterocolitica* and Gram-positive bacteria *B. cereus, B. subtilis, C. albicans, E. fecalis, M. luteus, S. aureus* and *S. epidermidis.* Result of

antimicrobial tests showed the essential oil was active only against Gram positive bacteria i.e. *B. cereus* and *S. aureus*; the extracts butanol was active against pathogen *P. aeruginosa* and *S. aureus.*

Basil leaf extract was most effective against all the bacteria isolated from bat's fecal matter while neem leaf herbal extract was least effective in all the herbal extract used i.e. neem leaf extract, citronella leaf extract, basil leaf extract and peppermint leaf extract reported by Singh (2019). In our results citronella oil was found most effective having maximum zone of inhibition (15mm) while eucalyptus oil was least effective against all the bacterial isolates.

Antimicrobial activity of antibiotics

The use of antibiotics (in clinical, veterinary and agricultural practices) generates selective pressure which is the major driving force leading to the emergence and spread of antibiotic resistance (Pallecchi *et al*. 2008). Determinants of antibiotic resistance in the wild environment present a serious menace to both human and animal vigor. The bidirectional potential for pathogen transmission between humans and wildlife has been shown by several studies (Schoub, 2012; Wheeler *et al*. 2012).

Antimicrobial susceptibility patterns were determined using commercial antibiotics procured from local market. The isolate HSb was resistant to cefexime with 25% concentration (0.01mg/µl) and susceptible to 50% (0.02mg/µl) and 100% (0.04mg/µl) concentration. Similarly, isolates HP6 and HP9 were resistant with 25% concentration against cefexime (0.01mg/µl) but sensitive to 50% and 100% concentrations. Isolates HSa, HAn, FR6, FR8 and HB9 were found resistant with 25% concentration against ampicillin (0.01mg/µl) and susceptible to 50% (0.02mg/µl) and 100% (0.04mg/µl) concentrations. HB7 was found to resistant to rifampin + isoniazid at 25% concentration (0.01+0.006mg/µl) but susceptible to 50% (0.02+0.01mg/µl) and 100% (0.04+0.02mg/µl) concentrations.

Rubinstein and Keynan (2013) found that *E. faecalis* strains have been reported as antibiotic resistant bacteria in many countries. It has been reported that *Enterococci* showed resistance against antibiotic such as erythromycin, tetracycline, clindamycin, beta-lactamases and vanomycin. Owusu-Kwarteng *et al.* (2017) evaluated that *Bacillus* strain generally resistant to Ampicillin, Penicillin, Cefexime, Amoxicillin and Oxacillin. They were however susceptible to other antimicrobials such as Chloramphenicol, Gentamycin, Rifampin, Tetracyclin, Vanomycin and Clindamycin. Similarly, in our present study isolate HP9 was found resistant against Cefexime.

Silva *et al.* (2013) found 15 genera of bacteria divided into Gram-negative (*Escherichia, Citrobacter, Klebsiella, Enterobacter, Serratia, Hafnia, Proteus, Providencia, Aeromonas, Pseudomonas* and *Acinetobacter*) and Gram-positive (*Staphylococcus, Streptococcus, Enterococcus* and *Micrococcus*). The antibiotic susceptibility test showed that the response to the antibiotics: cefepime, ceftazidime, amoxicillin, ampicillin, cephalothin, cefoxitin, cefotaxime, piperacillin/tazobactam, meropenem, gentamicin, amikacin, ciprofloxacin, sulfamethoxazole/trimethoprim, chloramphenicol and tetracycline was heterogeneous in most of the bacterial isolates. Results further revealed that among the isolates, *K. pneumoniae* and *P. aeruginosa*

showed resistance to most of antimicrobials. Meropenem was the only antibiotic effective on all the isolates. Similarly, our results revealed the different pattern of susceptibility of different isolates at different concentration and with different antibiotics.

In the present investigation it was found that *Pseudomonas* spp. showed susceptibility towards Chloramphenicol, Ampicillin, Cefexime, Ofloxacin and Rifampin + Isoniazid. Similarly, Arjun (2020) reported the susceptibility of *Pseudomonas* spp. towards antibiotics Cefexime, Chloramphenicol, Ampicillin, Ofloxacin and Rifampin+ Isoniazid. It was also reported by Neetu (2017) that *Pseudomonas* spp. sensitive towards the Cefexime, Chloramphenicol and Ampicillin/ Cloxacillin.

Antibiotic-resistant strains of coliform bacteria in the mollusc intestines was reported by Watkinson *et al.* (2007). Regarding the antibiotic susceptibility profile, Pidiyar *et al.* (2002) characterized *A. culicicola* from the midgut of *Culex quinquefasciatus* and observed that this bacterial species exhibited resistance to ampicillin and sensitivity to ciprofloxacin. In our present study there was not a single bacterium found to be multi drug resistant.

In the last few decades, so many plant species were explored for obtaining potential antimicrobials for therapeutic purposes (Rios & Recio, 2005), which later on become an integral part of primary health care in many parts of world (Cowan, 1999). Antibacterial activity of different solvent extracts viz., petroleum ether extract, methanol, chloroform, benzene and ethanol extracts of root of *Operculina turpethum* (L.) was tested against six bacterial species viz., *E. coli, Proteus vulgaris, K. oxytoca, B. cereus* and *S. aureus* at different ppm concentrations (Kiran *et al.* 2018).

Due to alarming increase in the incidence of new and re-emerging infectious diseases simultaneously development of resistance to antibiotic due to clinical use, a continuous and urgent need to discover new antimicrobial compounds with novel mechanism of action (Rajendran & Ramakrishnan, 2009), Plants are rich source of wide variety of secondary metabolites viz., terpenoids, alkaloids, tannins and flavonoids possessing enormous antimicrobial properties (Suresh *et al*.1992) and are capable of inhibiting or slowing the growth of bacteria, yeasts and moulds. Essential oils and their components have activity against a variety of targets, particularly the membrane and cytoplasm and completely change the morphology of the cells. Approximately 25 to 50 % of current pharmaceuticals are derived from plants, is found effective against many pathogenic bacteria (Bilgrami *et al*. 1992).

The prevalence of antibiotic resistance genes in the wild communities of wild fauna represents a potential danger for future disease control and animal treatment regimes. The loss of ability to successfully treat disease, such as risk of therapeutic failure due to resistance genes is detrimental to endangered species recovery programs (Deem *et al*. 2001).

CHAPTER-VI

SUMMARY AND CONCLUSION

The present investigation was carried out in Rodentology laboratory of department of Zoology and Aquaculture, CCS Haryana Agricultural University, Hisar for isolation and characterization of bacterial agents from sample of visceral mass or tissue from fresh water snails. These bacterial isolates were further tested against three essential oils and five antibiotics to determine their susceptibility. The results were observed and summarized here below:

- Total thirty-eight bacteria named as HSa, HSb, HSx, HSj, HAd, HAo, HAn, HAf, HB1, HB2, HB3, HB4, HB5, HB6, HB7, HB8, HB9, HB10, HB11, HB12, FR1, FR2, FR3, FR4, FR5, FR6, FR7, FR8, FR9, FR10, FR11, FR12, HP1, HP2, HP3, HP4, HP5, HP6, HP7, HP8, HP9 and HP10 were isolated from the collected sample during the study.
- The collection sites for fresh water snail were water canal (Satrod), water canal (Azad nagar), water canal (Balsamand road), rice fields (Fatehabad) and lotus pond (CCS HAU, Hisar).
- On the basis of morphological classification most of the bacterial colonies were found circular HSa, HSb, HSx, HSj, HAd, HAo, HAn, HAf, HB2, HB3, HB4, HB5, HB6, HB7, HB9, HB10, HB11, FR1, FR2, FR3, FR5, FR6, FR7, FR8, HP1, HP2, HP4, HP5, HP6, HP7, HP10; with entire margin HSa, HSb, HSx, HSj, HAd, HAo, HAn, HAf, HB2, HB3, HB4, HB5, HB6, HB7, HB8, HB9, HB10, HB11, FR1, FR2, FR3, FR5, FR6, FR7, FR8, HP1, HP2, HP4, HP5, HP6, HP7, HP10 and with convex elevation HSa, HSb, HSx, HSj, HAd, HAo, HAn, HAf, HB3, HB4, HB5, HB7, HB9, HB11, FR1, FR2, FR3, FR5, FR6, FR7, FR8, HP4, HP7 and HP10.
- Out of thirty-eight bacterial isolates twenty were Gram negative HSb, HSx, HSj, HAf, HB1, HB2, HB4, HB7, HB10, HB12, FR3, FR7, HP1, HP2, HP3, HP4, HP5, HP6, HP7, HP10 and other eighteen were Gram positive.
- Bacterial isolates HSa, HSb, HSx, HAd, HAo, HB1, HB2, HB3, HB5, HB6, HB8, HB10, HB11, HB12, FR2, FR6, HP1, HP2, HP5 and HP6 were found cocci shaped.
- All the bacterial isolates were found catalase positive except HB6 and HB8.
- Out of thirty-eight bacterial isolates nineteen were found oxidase positive HSb, HSx, HAd, HB1, HB2, HB3, HB5, HB10, HB11, HB12, FR3, FR6, HP1, HP2, HP3, HP4, HP5, HP6, HP10 and rest were oxidase negative.
- On the basis of primary and specific secondary biochemical tests the bacteria identified were related to different genera of *Staphylococcus* (Isolate I-III), *Neisseria* (Isolate I-II), *Yersinia*, *Micrococcus* (Isolate I-V), *Clostridium* (Isolate I-V), *Enterobacter* (Isolate I-II), *Campylobacter* (Isolate I-II), *Pseudomonas* species 1 (Isolate I-VI), *Pseudomonas* species 2 (Isolate I-III), *Proteus*, *Streptococcus*, *Enterococcus*, *Bacillus* (Isolate I-III), *Salmonella* (Isolate I-II) and *Aeromonas* species.

- The three essential oils (Eucalyptus oil, Citronella oil and Lemon oil) were tested for their antimicrobial activity against the isolated bacteria.
- The zone of inhibition was found to be decreased as when the concentration of essential oils decreases. The maximum zone of inhibition (10mm) was found in bacterial isolate FR8 by the Eucalyptus oil with 100% concentration (0.90mg/µl).
- Citronella oil was found most effective against HP4 with zone of inhibition (15mm) at 100% concentration (0.89mg/µl).
- Maximum zone of inhibition was seen in bacterial isolate HB2 by the Lemon oil at 100% concentration (0.85mg/µl) having zone of inhibition 14mm.
- The bacterial isolates HSb and HAn were found resistant with Eucalyptus oil at 25% concentration (0.22mg/µl) but sensitive to 50% (0.45mg/µl). and 100% (0.90mg/µl) concentrations. Similar pattern also showed by bacterial isolate HP3 which was resistant to the Citronella oil with 25% concentration (0.22mg/µl) but susceptible to higher concentrations.
- Both isolates FR4 and HB8 were found resistant with Lemon oil at all three concentrations i.e. 25% (0.21mg/µl), 50% (0.42mg/µl) and 100% (0.85mg/µl).
- Lemon oil was found most effective on all isolates except FR4 and HB8.
- All the bacterial isolates were also checked for antibiotic susceptibility test against five (Cefexime, Ofloxacin, Chloramphenicol, Ampicillin and Rifampin + Isoniazid) commercially available antibiotics.
- The bacterial isolates HSb, HP6 and HP9 were found resistant to Cefexime with 25% concentration (0.01mg/µl) and susceptible to 50% and 100% concentration.
- Similarly, Isolates HSa, HAn, FR6, FR8 and HB9 were found resistant with 25% concentration (0.01mg/µl) against Ampicillin and susceptible to higher concentrations.
- HB7 was found to resistant to Rifampin+Isoniazid at 25% concentration (0.01+0.006mg/µl) and susceptible to 50% and 100% concentrations.
- Cefexime, Ofloxacin, Chloramphenicol, Ampicillin and Rifampin+ Isoniazid were most effective to all other isolates.
- Not a single bacterium was reported to be multi-drug resistant.

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ABSTRACT

Title of Thesis	**:**	**Efficacy of essential oils against bacterial isolates from fresh water snails**
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Title of Degree	**:**	Master of Science
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Year of Award of Degree	**:**	2021
Major Subject	**:**	Zoology
Total number of pages in thesis	**:**	73 + x
Number of words in abstract	**:**	253

Keywords: Fresh water snail, Bacterial isolates, antibiotics, essential oils and antimicrobial efficacy.

The present investigation was carried out in CCS Haryana Agricultural University, Hisar and nearby selected sites in and around district Hisar (Haryana) for the isolation and characterization of bacterial agents from visceral mass or tissue of fresh water snails. During the course of study total thirty-eight bacteria were isolated from the fresh water snails collected from various ponds and water canals in and around district Hisar. On the basis of primary and specific secondary biochemical tests, *Staphylococcus* (Isolate I-III), *Neisseria* (Isolate I-II), *Yersinia*, *Micrococcus* (Isolate I-V), *Clostridium* (Isolate I-V), *Enterobacter* (Isolate I-II), *Campylobacter* (Isolate I-II), *Pseudomonas* species 1 (Isolate I-VI), *Pseudomonas* species 2 (Isolate I-III), *Proteus*, *Streptococcus*, *Enterococcus*, *Bacillus* (Isolate I-III), *Salmonella* (Isolate I-II) and *Aeromonas* related to different genera were identified and found more prevalent in collected samples. The bacterial isolates were analyzed for antimicrobial susceptibility to five antibiotics (Cefexime, Ofloxacin, Chloramphenicol, Ampicillin and Rifampin+Isoniazid) and three essential oils (Eucalyptus, Citronella and Lemon) at different concentrations. On the basis of antibiotic susceptibility test not a single bacterium was reported to be multi-drug resistant. All antibiotics are most effective against all bacterial isolates. Bacterial isolates FR4 and HB8 were found resistant with Lemon oil at all three concentrations viz. 25%, 50% and 100%. However, Lemon oil was found most effective against all the bacteria isolates except FR4 and HB8. Antibacterial activity of the selected essential oils was good against all the tested bacterial isolates. Thus, use of essential oils can help in diseases control and safe for environment due to its organic nature.

MAJOR ADVISOR **DEGREE HOLDER**

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- Qualify GATE 2021.

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(Jyoti Soni)